BONE STRUCTURE AND METABOLISM

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CIBA FOUNDATION SYMPOSIUM

ON

BONE STRUCTURE AND METABOLISM

Editors for the Ciba Foundation

G E W WOLSTENHOLME, *O B E* M A M B B Ch

and

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With 121 Illustrations



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PREFACE

THE Ciba Foundation, London, is an educational and scientific charity founded by a Trust Deed made in 1947 Its distinguished Trustees, who are wholly responsible for its administration, are The Rt Hon Lord Adrian, O M , F R S , The Rt Hon Lord Beveridge, K C B , F B A , The Hon Sir George Lloyd Jacob, Kt , and Mr Raymond Needham, Q C The financial support is provided by the world wide chemical and pharmaceutical firm which has its headquarters in Basle, Switzerland

The Ciba Foundation forms an international centre where workers active in medical and chemical research are encouraged to meet informally to exchange ideas and information It was opened by Sir Henry Dale, O M , F R S , in June 1949

In the first six years, in addition to many part day discussions, there have been 37 international symposia, each lasting two to four days, attended by outstanding workers from many countries Other symposia are planned at the rate of five or six a year

Acting on a suggestion, made a few years earlier, by members of the committee of the British Bone and Tooth Society, the Director found an opportunity to include a symposium on Bone Structure and Metabolism among the small international conferences which were organized at the Ciba Foundation in 1955 Information provided by the former honorary secretary of the Society, Dr T F Dixon now Professor of Biochemistry in Baghdad, and by the present honorary secretary, Dr H R Perkins, was of the greatest value to the Director in the arrangements for this meeting He also received much advice on membership and the scope of the sessions from Dr C E Dent, who later gave the symposium the benefit of his knowledgeable and benevolent chairmanship

The informality and intimacy of these meetings have permitted discussion of current and incomplete research and stimulated lively speculation and argument They have also been the occasion for reference to much published and unpublished work throughout the world The proceedings are issued in full, with only the minimum of editing, in order to pass on to a far wider audience the benefits of these meetings It is hoped that readers will not only gain information and inspiration from this report, but will also feel that they share in these frank and friendly discussions

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List of those participating in or attending the Symposium on "Bone Structure and Metabolism", 12th–14th July, 1955

R Amprino	Inst of Human Anatomy, University of Bari
W D Armstrong	Dept. of Physiological Chemistry University of Minnesota
L F Bélanger	Dept of Histology and Embryology, School of Medicine University of Ottawa
B de Bernard	Faculty of Medicine Paris, and Pavia
J A Black	Royal Hospital for Sick Children Glasgow
K L Blaxter	The Hannah Dairy Research Inst Kirkhill Ayrshire
M J Dallemagne	Inst of Experimental Therapy University of Liège
C E Dent	University College Hospital Medical School London
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A Engström	Dept of Physical Cell Research Karolinska Inst Stockholm
G Fanconi	Children s Hospital Zürich
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E RUTISHAUSER	Dept of Pathology University of Geneva
D D VAN SLYKE	Brookhaven National Laboratory, Upton, New York
J STANBURY	Massachusetts General Hospital Boston

CHAIRMAN'S OPENING REMARKS

C. E. DENT

DR WOLSTENHOLME in his opening words has covered very fully the procedure to be followed at our meetings during the next few days. Before going further I want to express our deep appreciation of his work in organizing this Symposium and our gratitude too to the Ciba Foundation by whose generosity it has been made possible. Many of us here have experience of the vast amount of work required in order to plan and bring about representative international meetings of this kind, and it is a real pleasure, mixed with feelings of relief, to recall the manner in which Dr Wolstenholme and his staff have shouldered this burden for us.

You will note, on turning to the programme, that the first papers concern the more fundamental approach to the structure and metabolism of bone. From this we proceed through biochemistry and then physiology, and finally come to rest with clinical medicine. This is a most logical and suitable arrangement of subject matter. It puts first things first and reminds those of us who deal with the interesting immediate applications of knowledge, that we are always ultimately dependent on the basic scientist, whose work we must make serious efforts to comprehend, even if it may appear at first sight to be somewhat remote from ordinary affairs.

Between us we cover very many aspects of our chosen subject. We each know a great deal about certain narrow aspects and are painfully aware of our ignorance of almost everything else. I hope that in the discussions we will not allow such ignorance to cramp our style in any way on the mistaken assumption that we should only talk about things we fully

understand May I suggest also that in the next few days we act not so much as members of a Symposium—this word is rather too formal for my liking—but as simple members of a family gathering, brought together from all parts, many of us strangers, but all of us already closely knit by a common loyalty and ideal

STRUCTURE OF BONE FROM THE ANATOMICAL TO THE MOLECULAR LEVEL

ARNE ENGSTRÖM

Department of Physical Cell Research, Karolinska Institute, Stockholm

FROM a general physiological point of view the skeleton was for a long time considered to be of minor interest. However, with the introduction of radioactive isotopes to the study of the metabolism of the calcified tissues the utterly labile state of the mineral salts was clearly demonstrated and it was early shown that spongious bone had a higher "turn over" than compact bone. The rate of rebuilding is thus higher in the spongious than in the compact bone, a finding which is also verified by histological examination. This communication will try to give some general aspects on the biological role of the rebuilding of bone seen from the macroscopic through the microscopic down to the molecular level.

The gross shape of the bones reflects their supporting function and the response of the compact and spongious bone to mechanical forces has been extensively investigated. Recently Hirsch (1955, personal communication) has applied modern measuring techniques to this problem. In autopsy specimens the neck of human femur was dissected free and the shaft of the bone was firmly attached in a special holder. A load was applied to the top of the caput (curves A, B, C and D in Fig. 1 indicate various positions of the load) and the signals from strain gauges placed on the lower side of the neck were recorded. The interesting observation was made that the deformations of the neck were not very much changed if the spongious bone inside the neck was removed. If small incisions were made in the cortical bone, however, the deformation was great. This experiment therefore showed that the spongious

bone did not contribute greatly to the mechanical stability in this particular case

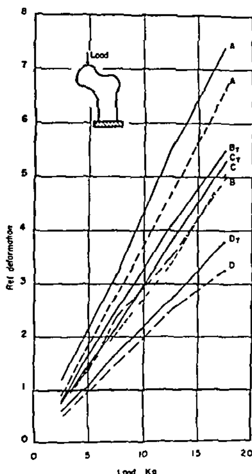


FIG 1 The deformation of the neck of the femur with (index T) and without spongiosa The letters A B C and D indicate various positions of the load in the two cases Removal of the spongious bone does not influence the deformation to any considerable extent (Reproduced by courtesy of Dr C Hirsch)

It is the highly organized system of organic inorganic material which gives the great stability of the bone tissue and

a study of the quantitative distribution of the mineral salts within the histological units composing the bone tissue gives information of great interest The distribution of mineral salts in osseous tissues has been examined by the methods of quantitative X ray microscopy developed in our laboratory (for references see Engström, 1946, 1953, 1955) The advantage of utilizing X rays for microscopy is that the X ray absorption can easily be correlated to the chemical composition of the specimen The following procedure has been adopted for the study of bone tissue Sections of a few hundred microns or less in thickness were prepared from bone tissue by grinding These sections were placed in contact with a fine grained photographic emulsion (Eastman Kodak Spectroscopic Plate 649 or Kodak Maximum Resolution Plate) and an image of the ground section was recorded with soft X rays The wavelength of the X rays has to be chosen so that all absorption in the image is caused by the mineral salts A microradiogram of the decalcified specimen registered under the same conditions should give no contrast at all The resolution of the small X ray image on the fine grained photographic emulsion is so high that it can be examined under high power in the optical microscope It is possible to record X ray images (microradiograms) which have a resolution of 0 25 μ and the ultimate limit of resolution is the resolving power of the light microscope Differences in the degree of mineralization will show up as differences in photographic density and a microphotometric evaluation of the densities will give the local amounts of absorbing substances in this case the amount of inorganic material The theoretical basis for this technique is given elsewhere (Engstrom, 1946, 1953, 1955, Engfeldt and Engstrom, 1954)

The microradiographic study of the mineral distribution in bone tissue revealed that the content of mineral salts varied from one structure to another Amprino and Engstrom (1952) showed that there was a progressive increase in the mineralization of the osteons (cf Fig 2) and that the number of lowly mineralized structures was greater in spongious bone than in

the compact bone (Fig 4) These findings were entirely verified by the application of micro interferometry to thin sections of bone tissue (Fig 3) (Davies and Engstrom, 1954)

The relative proportion of inorganic and organic material in the various structures of bone have been studied by micro interferometry (Davies and Engstrom, 1954) and by X ray microradiography After decalcification a microradiogram taken with X rays of much longer wavelengths (8–10 Å) with special equipment (Engstrom and Lindstrom, 1950, Combee and Engstrom, 1954) shows the distribution of organic material (Fig 7)

It was observed by Borst and Konigsdorffer (1929) that in the classical case of porphyria (Petry) the porphyrins were localized in certain Haversian systems, indicating varying

PLATE I

Fig 2 Microradiogram of a 200 micron thick ground cross section of the femur of a 7 year old boy The microradiogram was registered with 35 kv X rays on a Maximum Resolution Plate showing the varying degree of mineralization White structures have a high content of mineral salts The osteocytes appear as small black oval dots with no minerals The organic content of the Haversian canals shows no absorption of the 35 kv X rays

Fig 3 A 5 micron thick ground section photographed in a Cook Troughton and Simms interference microscope The shift of the interference fringes indicates that the Haversian system in the centre has less content of minerals than the surrounding bone tissue which is in accordance with the mineral distribution shown in Fig 2

Fig 4 Microradiogram showing the mineral distribution in the spongious bone tissue in caput femoris from an adult human The abundance of lowly mineralized areas is well demonstrated 40 kv X rays Eastman Kodak Spectroscopic Plate 649

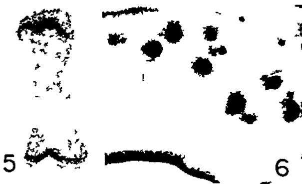
Fig 5 Radioautograph of a part of a rat tail The animal had received radioactive strontium 8 days before being sacrificed The high uptake of ^{90}Sr is clearly seen

Fig 6 Radioautograph of a ground cross section of a femur from a dog which had received ^{90}Sr one week before being sacrificed Note the high uptake in the endosteal layer and in certain Haversian systems The microradiogram of the same section showed that the isotope labelled systems had a low content of mineral salts

Fig 7 Microradiogram of 5 micron thick section of a decalcified human rib This picture was recorded with 1 8 kv X rays on Lippmann emulsion in order to show the distribution of mass It is evident that the organic substance is evenly distributed in the Haversian systems and also the content of organic material in the Haversian canals as well as the bone marrow is clearly seen The amount of organic substance is high in the cementing lines











Figs 2-7

[facing page 6

physicochemical properties of the different histological structures One could therefore suspect that isotopes of calcium or phosphate, when introduced into the organism, would also show a specific pattern of localization and it was shown by Engfeldt, Engström and Zetterström (1952) that radio active phosphate was concentrated in the adult bone tissue mainly in those structures which had a low content of mineral salts i e in structures in the process of being mineralized The uptake of isotope in the spongious bone is therefore high, as is demonstrated by the radioautograph in Fig 5 It is of interest in this connection to note that a great number of radioactive products appearing at fission of heavy atomic nuclei are bone seeking and thus will become localized in the lowly mineralized structures in bone where they give rise to internal radiation damage Fig 6 shows how ^{90}Sr is deposited in such "hot spots" in compact bone tissue

The lowly mineralized structures in bone tissue are thus in a state of very rapid exchange with body fluids, a property which indicates the physiological function of these structures In fact, the mineralized tissues in the body act as an effective ion exchange column

The experimental findings reported above indicate that there must be a molecular organization of the mineral salts in the bone tissue which is particularly well suited for ion-exchange mechanisms Recent X ray crystallographic studies on bone (Finean and Engstrom, 1953, Carlstrom and Finean, 1953, Carlstrom, 1955) have shown that the crystallites of the bone salts have the dimension 220 × 65 Å There seems to be a close relationship between these crystallites and the collagenous fibres The inorganic crystallites are aligned with their long axes parallel to the collagen fibres and three inorganic crystallites seem to fill up one "period' of the collagen

There has been a lengthy discussion on the chemical nature of the bone salts and various types of calcium phosphates have been proposed In discussing the natu e of the bone salt one must always remember the minute size of the crystallites which means that they have a large surface area One

gram of bone salt has an area of 130 square metres Carlstrom (1955) has discussed these problems and among the proposed calcium phosphates in bone tissue the only crystallographic ally possible compound is hydroxyapatite As mentioned above the crystallite size is small and therefore the large surface area can adsorb various other ions The calcium carbonate in bone most probably exists in a form adsorbed on to the surface of the apatite The small size of the carbonate explains why no calcite lines appear in the X ray diagrams Carlstrom (1955) heated bone in an atmosphere of carbon dioxide at 800° C for a long period and the carbonate crystal lites increased in size so that it was possible to record the X ray reflections from calcite superimposed on the hydroxyapatite pattern

The width of the apatite crystallites corresponds only to about seven unit cells (the unit cell is $a = 9$ 42 and $c = 6$ 88) and therefore the relative number of groups in the surface position will be comparatively large Fig 8 shows diagram matically the appearance of the 100 surface whether ended in an acid or basic medium It is natural that the adsorption of, for example, calcium or phosphate on to the surface of the small crystallites will greatly influence the net chemical composition of the bone salt Large crystallites of apatite, for example prisms with a diameter of 3000 Å, would have a molar Ca/P ratio of 1 667, and the adsorption on to the surface of calcium or phosphate exclusively would influence this value only to a very small extent In the case of the small crystallites found in bone however the surface bound ions will greatly influence the Ca/P ratio depending on whether the crystallites end up with calcium or phosphate (electro *neutrality will be accomplished for example by hydronium* or hydroxyl ions) If a 50 Å thick crystallite has all three possible surface positions filled with calcium the Ca/P ratio will be close to 2 and if instead two phosphate groups make the excess on the surface the Ca/P ratio will be between 1 3 and 1 4 It is evident as Carlstrom (1955) has pointed out that chemical analyses of bone salt and synthetic apatites which

have small crystallite sizes are of little value in the interpretation of their crystallographic structure, and it is not necessary to assume whether they are "defect" or substituted apatites in order to explain the chemical and physical properties of the "colloidal calcium phosphates"

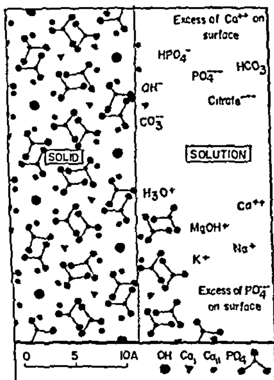


FIG 8 A simplified drawing of the (100) surface of hydroxyapatite seen in the direction of the c axis Some of the ions known to be bound at the surface of the bone crystallites are indicated in the solution (Carlstrom 1955)

The considerations presented above indicate that the bone tissue, seen from the molecular to the histological level, has the characteristics of a system which exerts the *most intimate* contact with the tissue fluids and that changes in the surface composition of the crystallites can take place at various places at a very high rate This is one of the important physiological

functions of the bone tissue, and when applied to various pathological conditions these aspects have given new views on a number of hitherto obscure conditions The biological drawback of these quick exchange reactions is that harmful products, also, such as the components appearing after atomic explosions, will be adsorbed and localized in the skeleton (Engfeldt *et al*, 1954) The obviously great physiological role of the skeleton in normal and diseased conditions and the increased risk for the human individual of acquiring skeletal "infections" with radioactive fission products, necessitates further intensive research on the mineralized tissues

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DISCUSSION

Armstrong Robinson measured the length of bone particles and got a figure rather like your 660 Å You indicated that three of your crystals were put together to make one particle that he sees on the electron microgram Would you expand a little more on how three crystals make one particle?

Engström We have not done any electron microscopy on bone If you take a piece of fish bone and heat it slowly, you will get a meridional diffraction pattern with spacings of 220 Å, 440 Å and 660 Å If you take away the collagen you get the same spacings, which could be interpreted as indicating some sort of interruption in the crystals, at least at the longest spacing If you heat bone slowly you will find that the 220 Å spacing diminishes in intensity and only the longer spacings remain There may be some intermediate material which can fuse the crystallites together—it may well be carbonate—I would not be surprised if we are unable to distinguish in the electron microscope between carbonate and hydroxyapatite and therefore see larger aggregates with the latter technique

Armstrong His width was also a little wider than three times your 70 Å

Engström Robinson and Watson show plate like structures which are about 50 Å in one dimension and 200–300 Å in the other As we get a straight curve relating the low angle scatter with scattering angle we think that there is a fairly symmetrical cross section of the particles The length of the particles may be of the order of 200 Å A plate can easily be built up by aggregation of such particles Plates have been described in preparations of synthetic apatites which by X ray diffraction are *not* apatites These plates are some sort of a two dimensional pseudoapatite' with spacings which do not exist in bone apatites

Follis What does this third dimension look like? Is it round?

Engström We think it is a fairly symmetrical cross section, that is all we can say at present It may be a hexagonal prism or perhaps a little more irregular This is from X ray diffraction evidence

Lacroix Prof Engstrom what is the difference at the molecular level between the fully calcified and the less calcified osteon?

Engström That is a difficult question to answer, because the particles in mature bone are already so small that they give poor X ray diffraction, and if they were smaller we would not see much of them in this sort of X ray investigation It has been postulated that the apatite particles are swimming in an organic medium but I would say, from the figures we gave, that we have roughly 40 per cent by volume of inorganic and 60 per cent organic it must be a rather close packing in fully calcified bone

Blaxter How does modification of the crystal surface alter the composition of the interior of the crystal? Do you get recrystallization?

Engström We think that the nucleus of the crystallite is hydroxyapatite and the changes or modifications take place on the surface If you try to make synthetic hydroxyapatites and try to introduce Mg or perhaps more correctly Mg(OH) the growth of the crystallites is prevented

Rogers Could you tell us a little more about the endosteal region? We (Rogers H J Weidmann S M and Jones H G (1953) *Biochem J* 54, 37) have found that the ^{32}P content of this part of the femur is very high compared with the rest of the bone It is important to I now whether this large uptake of isotope *in vivo* is due entirely to the presence of young osteons

Engstrom We think that it is young osteons which have a high uptake of isotopes but Prof Lacroix will say that there seem to be a few exceptions We have not done the quantitative correlation between the amount of isotope and degree of mineralization, only by visual comparison Quantitative data are being assembled

Bélanger In 1953 we published (Bélanger L F (1953) *J dent Res* **32**, 168) a picture which indicated that this seems to be the case It showed the isotopic exchange at the level of mineralized bone after soaking in a solution of ^{32}P We expected to find most of the exchange taking place in the diaphysis which is the most highly mineralized portion of the bone on the contrary we found exchange taking place mostly in the epiphyseal areas which are the least mineralized, so this seems to indicate that these least mineralized areas are more easily accessible for transit and consequently for isotopic exchange

Lisco What animal was this and what was the age of the animal?

Bélanger This was a growing male rat of about 80 g This was a section of mineralized bone placed in a solution of phosphoric acid for an hour

Lisco I suppose you would expect a correspondence between this ^{32}P and ^{45}Ca if you did it the same way *in vitro*?

Bélanger I expected it but it does not work that way there are complications which I may perhaps discuss at length later

Dallemagne Prof Engstrom what do you think about a bond between the organic and the inorganic matter of bone?

Engstrom The mere fact that the components are so closely tied up is I think an indication of some sort of a bond between them but we do not know the exact nature of that bond If we could get some *in vitro* system working and block certain organic groups we might learn more about the relationship between the organic and inorganic phases of bone

Dent What is the function of these crystals? Is it correct to imagine that the bone is being strengthened by little objects which stop the sort of shearing movements that might occur in an otherwise rather flexible organic matrix? Is it a sort of reinforced concrete?

Engstrom Yes I think Prof Lacroix has once mentioned prestressed concrete The work which Amprino and we have done together shows that lowly mineralized bone is not very stable mechanically but has a high degree of ionic exchange

Nordin Does your work on the stressing of the head and neck of the femur mean that all that we have learned about the lines of stress and the way the collagen fibrils lay themselves along just the right lines according to the stresses is all misleading and we have got to discard it?

Engstrom No I would not be so brave It merely indicates that in this case the total mechanical stability the major part I would say nine tenths is given by the outer zone of the femur neck

Rutishauser I think that these observations concern the really acute form of stress Chronic bone stresses (30–60 days in dogs) affect the cortex at the point of maximum tension rather than the centre initially

Lacroix A recent monograph from my laboratory (Vincent 1955) contains an illustration showing a difference in Ca content from one

lamella to the other This difference is not seen—at least not with the kind of soft X ray that has been used—in a very young osteon, and it fades out when the full load of Ca is reached

Engström You see the same thing in the interferometer microscope

Nordin If you decalcify the neck of the femur so that nothing is left but the organic phase does it just collapse or has it got any strength left? Has the matrix got any strength in its own right?

Engström I have not done that, but I imagine it may collapse

Armstrong Is it not a fact that the inorganic material of bones is located in relation to the 640 Å spacings of the collagen fibrils?

Engström I think there are indications of that both from electron microscopy and X ray diffraction studies

Armstrong This observation does not prove the point but it does suggest that there may be some association between the organic fibrils and the inorganic material

Meyer Does the collagen fibril in bone have homogenous distribution of width or not? I would assume that if the width were variable you would get only a statistical distribution not along the long axis but along the short axis the *c* axis

Engstrom The width of the fibrils varies quite a lot but the spacing seems to be fairly constant I have no idea about the distribution of the widths in bone collagen

STRUCTURE OF BONE SALTS*

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IT is remarkable that since De Jong (1926) established that bone salts have a molecular structure similar to that of apatite numerous studies dealing with the same subject have still not achieved completely satisfactory results. However, at first sight the problem appears simple: bone salts† mainly contain calcium, phosphorus, carbonic anhydride and water. The point is to establish their reciprocal chemical relations and to determine their molecular structure. Thereafter, the position of the secondary elements, magnesium, sodium and potassium, still has to be defined.

Mineralization of bone salts

The study of bone salts requires the destruction of the organic part of bone and this is the first serious difficulty. For 50 years Gabriel's method (1894) has been commonly used, but what is the effect of such treatment on the molecular structure of bone salts? The question has already been put clearly by Armstrong (1950). The principal argument in defence of this method is that the X-ray diffraction pattern of

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† In the following pages we refer to tricalcium phosphate hydrate as TCPH, to glycol ashed bone as bone salts and to the quantity of calcium which raises the Ca/P weight ratio of bone from 1.94 (TCPH) to 2.26 as excess calcium.

total bone does not seem to be affected by glycerol KOH action But the breadth of the X ray diffraction lines of glycol ashed bone and total bone hinders any precise analysis of the radiograms, which reduces the value of the argument (Carlström, 1955) Nevertheless, it can be assumed that both structures are roughly the same It has been stressed that different results are achieved when either total or glycol ashed bone are analysed (Burns and Henderson, 1935), but the fact does not seem conclusive, because glycol ashing may have eliminated mineral elements which have no connection with bone salts proper

The other mineralization methods, ethylene diamine, tryptic digestion or ignition, alter the composition or structure of bone salts more strongly Gabriel's method is most certainly open to criticism, but alone it does not give rise to gross apparent modifications, we have to use it because it is less injurious than the others

We must point out that if the organic and mineral parts of bone are bound together, the destruction of the first must necessarily induce some changes in the composition of the second

Finally, if glycol ashed bone does not exactly represent the mineral part of the skeleton, study of its composition and properties was at least a fruitful approach to the problem and contributed to progress in the chemistry of calcium phosphates

Binding between carbonate and phosphate phases of bone salts

Previous experiments (Dallemagne and Brasseur, 1947) showed that the carbonate and phosphate phases of the bone salts are chemically independent We considered that a series of arguments, though open to criticism when taken separately had some value once they were put together, we recalled them several times (Dallemagne, 1951) Bone salts no doubt contain an appreciable amount of $CaCO_3$ though $CaCO_3$ lines are not seen on the X ray diagrams of bone salts But this does not necessarily favour a single phase hypothesis

(according to the conceptions of either Gruner and McConnell (1937) or Borneman Starinkevitch (1938)) Indeed, carbonate, adsorbed as a very dispersed phase on the surface of phosphate microcrystals, has no influence on X ray diagrams In favour of the two phases hypothesis, it must be stressed that the carbonate content of bone salts can be extracted specifically enough by hydrochloric acid and that bone carbonate decomposes in the same way as $CaCO_3$ during thermal treatment

The appearance of the CO_2 thermal extraction curve of bone salts is similar to the $CaCO_3$ decomposition curve, any small differences can be easily interpreted (Fig 1) Some bone CO_2 is eliminated as early as 300° C This probably corresponds to decomposition of $MgCO_3$ Most of the CO escapes between 500° and 600° C, as happens with pure synthetic $CaCO_3$ but the last traces of CO_2 are eliminated only around 700°–800° C This delay probably results from part of the CO_2 being trapped

Infrared spectroscopy studies provide a new argument in favour of the two phases hypothesis The typical lines of the bindings Mg—CO_3 and Ca—CO_3 appear on the patterns (Posner and Duyckaerts, 1954), but this does not seem to be absolutely conclusive, this method gives no quantitative results and is so sensitive that a very small quantity of adsorbed $CaCO_3$ and $MgCO_3$ can be detected, even if the greater part of these compounds is bound in some way to the fundamental phosphate crystals

All these arguments show that CO_2 is not fixed to the bone salts lattice by the same bindings as in mineralogical carbonate apatites But the possibility of binding by chemisorption at the surface of the phosphate microcrystals is in no way precluded

Hydroxyapatite

We think, therefore, that bone salts are not identical with mineralogical carbonate apatite just as we always opposed the opinion that they are composed of hydroxyapatite with

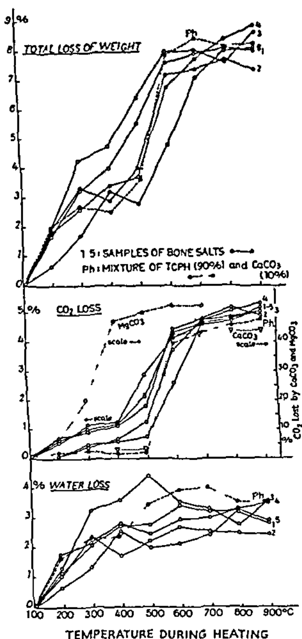


Fig 1 Effect of heating on bone salts (five different samples) on a mixture of TCPH (90 per cent) and $CaCO_3$ (10 per cent) on pure $CaCO_3$ and on pure $MgCO_3$ For each point 500 mg samples are used and heated during 24 hours The water loss value is calculated by subtracting the CO_2 loss (obtained by CO_2 determination) from the total weight loss

additional $CaCO_3$ Neither did we ever agree with the idea that TCPH and hydroxyapatite are similar We have no intention of entering into all these arguments again, we shall only deal with two of the most important points

We consider that bone salts cannot be hydroxyapatite, since the calculation of equivalence between cations and anions absolutely opposes this conception, should the fundamental molecule of bone salts be an hydroxyapatite, then they would not contain enough calcium to fix the CO_2

Neuman himself (1953) put forward an important argument against the hypothesis (Tromel and Moller, 1932) that TCPH is an hydroxyapatite which has adsorbed enough PO_4 ions to lower its Ca/P ratio from 2 14 to 1 94 This author established that tricalcium phosphate precipitates have an effective surface which is too small to fix enough PO_4 ions

Moreover, we consider it important as well as desirable not to define as "hydroxyapatite" molecules which differ from hydroxyapatite in most of their properties To avoid confusion, Fabry (1954) introduced the name "pseudoapatites" to describe synthetic calcium phosphates and bone salts which are both hydrated salts

Binding between excess calcium and TCPH

Since 1952, our conceptions have somewhat changed, owing to some disconcerting experimental results If it is true that the excess calcium of bone salts is present as $CaCO_3$ and that this is adsorbed on the surface of the phosphate microcrystals, an increase in the size of these microcrystals must liberate it Now we know that heating at an appropriate temperature causes these particles to increase in size With Posner, we established that the surface of crystals of bone salts dried at 105° C is 129 m^2/g and after ignition at 600° C is 60 m^2/g

Thus, we performed the following experiments samples of bone salts are heated for 24 hours at 100° 200°, 300°, 400° 900° C and then immersed in CO_2 free distilled water Alkaline elements are progressively liberated and titrated

with HCl and phenolphthalein, thereafter, the dissolved elements are chemically determined The 600° C and 700° C samples have the greatest alkalinity At these temperatures, the surface of the particles has shrunk to half of its original size and $CaCO_3$ has become CaO, much more soluble in water If the hypothesis that calcium carbonate is adsorbed on the surface of the phosphate microcrystals is true, a high enough proportion of CaO should be dissolved in the water phase But the calcium concentration in water always remains smaller than expected the largest amount found in water never exceeds 0 5 per cent of the total bone salts (i e little more than 3 per cent of the excess calcium), but we find that 60 per cent of the magnesium and the whole of the sodium is liberated The amount of calcium in solution is much smaller than the 14 per cent supposed to be adsorbed The most convincing interpretation of these experimental results is the following the excess calcium is bound to the phosphate by other forces than those of physical adsorption, a very small part of bone calcium is adsorbed on the surface of the crystals, one third of the magnesium replaces calcium in the phosphate molecule the remaining two thirds are adsorbed on the surface, sodium is located on the surface only and liberated as soon as the particles increase in size (Dallemagne, Fabry and Posner 1954)

Another series of experiments supports the import of these results

Fabry (1954, 1955) established (Fig 2) that pure synthetic TCPH or any pseudoapatite with a low Ca/P ratio, dried at room temperature, fixes calcium ions when immersed in a lime solution, 2 m equiv Ca/l (as shown by conductivity measurements) The Ca/P ratio of the solid phases increases with time, up to a constant value of 2 26

The higher the temperature, the more rapid is this calcium fixation The fixed calcium is not eliminated by washing with warm water, the fixation is an irreversible process Larger quantities of calcium can be fixed on the solid by using concentrated lime solutions, so as to reach a Ca/P ratio higher

than 2 26, but elution with warm water removes part of the calcium until the Ca/P ratio is reduced to the value of 2 26 Thus, this Ca/P value seems to constitute a quite characteristic limit It is remarkable that 2 26 is also the most frequently observed Ca/P value of bone salts

Therefore, the excess calcium is not simply physically adsorbed on the surface of TCPH microcrystals, but is bound

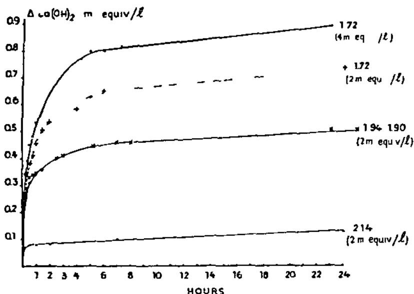


FIG 2 Calcium fixation on synthetic calcium phosphates with varying initial Ca/P weight ratio ranging from 1 72 to 2 14 m equiv /l of $Ca(OH)_2$ (determined by conductivity measurement at 25 C) fixed on 140 mg of solid immersed in one litre of a 2 m equiv /l lime solution

by other forces different from Van der Waals s and more probably corresponding to a chemisorption process This calcium can be considered to bind together the PO_4 and CO_3 groups

This conclusion necessarily gives rise to a new problem to determine what is the right kind of binding between excess calcium and TCPH and whether or not it differs in both pseudoapatite and mineralogical apatites There are strong grounds for thinking that the kind of binding is different here,

in fact, lies the crux of the problem of the molecular structure of bone salts

The concept of calcium atoms binding together TCPH and CO_3 fits in quite well with the fact that the $CaCO_3$ lines are not visible on X ray diagrams of bone salts Therefore, two calcium atoms must be linked to one CO_3 group Indeed, the calculation of equivalence between anions and cations gives better results when the following carbonates are supposed to be present in bone salts —Ca—$\left(\frac{CO_3}{2}\right)$, $MgCO_3$ (adsorbed), $NaHCO_3$ (adsorbed)

Calcium isotope studies

Falkenheim, Underwood and Hodge (1951) established that glycol ashed bone can exchange about 14 per cent of its calcium atoms when immersed in a solution of $^{45}CaCl_2$ On the other hand, Minder and Gordonoff (1953) showed that bone salts and the corresponding TCPH + $CaCO_3$ mixture give an exchange reaction but only to a very small extent when these materials are calcined, or when Norwegian natural apatite is used

Using a solution of $^{45}CaCl_2$ containing 5 m equiv Ca/l, we also found that equilibrium is reached after about 20 hours between the solution and glycol ashed bone (Fig 3), at this time, 14 per cent of calcium has been exchanged Following the agreement of our figures and Falkenheim's, we came to the conclusion that excess calcium represents 14 8 per cent of the total calcium of bone salts (for a Ca/P ratio of 2 26) On the other hand, we observed only a negligible exchange when using TCPH under the same experimental conditions We concluded that excess calcium only is involved in the exchange process This process is rather rapid and really consists of a true exchange (Dallemagne Fabry and Bodson, 1955*a*) Another experiment confirmed these views

We know that small quantities of HCl can selectively dissolve calcium bound to CO_2 A sample of bone salts which has previously exchanged 14 per cent of its calcium is

treated with different solutions of HCl (containing from 0 04 m equiv to 1 25 m equiv HCl/100 mg of bone salts) After each treatment with HCl (upper tracing of Fig 4) the

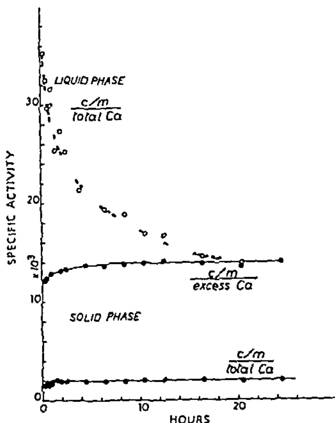


FIG 3 Specific activity variations of both solid (bone salts) and liquid phase ($^{45}CaCl_2$ + $CaCl_2$ 5 m equiv /l) Equilibrium is attained after about 20 hours The specific activity of the solid phase reaches the same value as the liquid phase when related to the excess calcium of bone salts

specific activity related to the excess calcium remains constant both in each liquid and in each residual solid phase This means that HCl rather selectively eliminates the excess calcium and that the same thing happens with radioactive calcium Therefore excess calcium coincides with exchanged calcium (Dallemagne, Fabry and Bodson, 1955b)

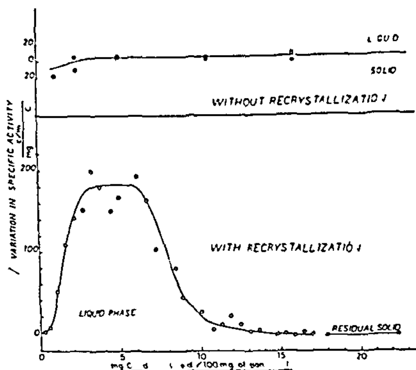


FIG 4 Percentage variation in specific activity of the hydrochloric acid fractions and of the residual solid (bone salts exchanged with ^{45}Ca) plotted against the quantity of dissolved Ca Above without partial recrystallization below with partial recrystallization in the solid during the exchange

o Values obtained in the liquid phase
● Values obtained in the solid phase

True exchange and recrystallization

Neuman (1950) and Falkenheim, Underwood and Hodge (1951) described the kinetics of the P and Ca exchange reactions the first rapid process is the true exchange, the second and slower process depends on recrystallization

The true exchange process is reversible, as demonstrated by Neuman and recently confirmed by us It probably consists of substituting by ^{45}Ca the calcium atoms of bone salts, which can be ionized when immersed in water But, under certain conditions, the rapid true exchange process is followed by a slower phenomenon described by Neuman as well as by

Minder and Gordonoff Then recrystallization is concerned We observed that such recrystallization takes place as soon as the $CaCl_2$ concentration of the radioactive solution is high enough Indeed, it takes place very easily and appears in many different circumstances, for example, in moist bone salts put in the oven at 105° C for drying, boiling in water for a few

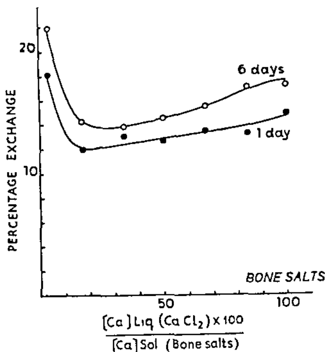


FIG 5 Percentage exchange of bone salts plotted against the $CaCl_2$ concentration of the liquid phase

hours has the same effect Under these conditions particles of bone salts increase in size, as shown by X ray diffraction Then the radioactive calcium exchange conditions are greatly disturbed True exchange slows down, ionization of the excess calcium decreases at the same time as ionic concentration increases in the liquid phases The reversibility of the process is reduced Fig 5 shows the influence of CaCl concentration of the liquid phase on the percentage exchange of bone salts the starting point (left) of this experiment is still higher than

14 per cent, which is only obtained with lower $CaCl_2$ content of the liquid phase. This percentage exchange increases slowly when the contact of the solid and the liquid phases becomes longer. On the other hand, recrystallization seems to block the exchangeable calcium atoms. If recrystallization has occurred during the contact of bone salts with the $^{45}CaCl_2$ solution, the results obtained when the solid is treated with HCl are quite different. Recrystallized samples of bone salts are submitted to thirty successive HCl fractions (0.02 m equiv./100 mg. of solid). As shown by the lower tracing of Fig. 4, the specific activity of the liquid phases (calculated by means of the formula $\left(\frac{\text{c.p.m.}}{\text{mg. excess Ca}}\right)$ is no longer constant but increases when HCl dissolves both the $CaCO_3$ fraction of bone salts and the recrystallized phosphate fraction. It becomes constant again when this fraction has been removed and becomes similar to the specific activity of the residual solid calculated by means of the same formula.

This recrystallization process, the nature of which is not well known, is important, as pointed out by Neuman, Toribara and Mulryan (1952), since it has some influence on the incorporation of radioactive elements in the skeleton *in vivo*. It explains why ^{45}Ca, after penetrating the skeleton, remains there for a very long time, which corresponds to the lifetime of the microcrystal in which it is fixed.

Bone salts and synthetic phosphates

On the basis of previous results, Posner, Fabry and Dallemagne (1954) elaborated a plausible theory concerning synthetic phosphates.

The following precipitates can be obtained by mixing suitable proportions of phosphoric acid and solutions of lime:

(1) Arnold's octophosphate, containing 8 Ca atoms/6 P atoms with a Ca/P weight ratio of 1.72.
(2) TCPH: 9 Ca/6 P (Ca/P 1.94).

(3) A more basic compound 10 Ca 6P (Ca/P 2 14), these figures are characteristic of hydroxyapatite (= precipitated hydroxyapatite of Wallaeys (1952))
(4) A still more basic compound 10 5 Ca/6 P (Ca/P 2 26)

All these substances have this in common, that they diffract X rays in an apatite like manner, they have the same hexagonal lattice

When calcined, the first two compounds give the X ray pattern of β tricalcium phosphate, the last two always retain the apatite structure, but thermal treatment enormously increases the crystalline particle size

When mixing lime and phosphoric acid solutions with a higher phosphorus proportion than is required by the 8 Ca/6 P ratio, the precipitate is no longer octophosphate, but brushite, which has no place in our series of phosphates since its X ray pattern is not of the apatite type On the contrary, with a large excess of lime, the precipitate is a basic phosphate with a Ca/P higher than 2 26, because, in addition, some lime is fixed by physical adsorption

The Ca/P values of 1 72 and 2 26 are therefore the extreme limits of the series of the synthetic apatite like calcium phosphates We must point out that precipitates with any Ca/P value between 1 72 and 2 26 can be obtained by working at random within these limits Therefore, it is a continuous series every term of which has the same crystalline lattice as apatite and contains the same number of phosphorus atoms per unit cell, but not the same quantity of calcium

However, synthetic calcium phosphates are different from mineralogical apatites, Fabry (1954) called them "pseudo apatites" We explain that pseudoapatite can have a Ca/P ratio lower than 2 14 by the postulate that calcium atoms are statistically missing in the structure They are "defect" compounds * We provisionally consider that pseudoapatite with the Ca/P of 2 14 represents the perfect crystalline

* NOTE ADDED IN PROOF As will be published later the defect pseudoapatite contains more water than the saturated compound

structure because crystallographic data show that the perfect apatite lattice contains a number of calcium and phosphorus atoms corresponding to this value However, pseudoapatites can have a Ca/P of 2 20 This is the maximum that can be reached It is difficult, at present, to give a satisfactory answer to the question about the position of this extra half atom of calcium However, the exchangeability of this supplementary half atom of calcium seems to be exactly similar to that of the tenth calcium atom

Bone salts correspond to a saturated pseudoapatite—even supersaturated with reference to classic crystallographic data—that is to say, to the upper term of the pseudoapatite series, containing 10 5 calcium atoms per 6 phosphorus atoms

Pseudoapatites and mineralogical apatites differ in several properties The water present in these compounds is most important the water of constitution and the hydration layer seem to be characteristic of pseudoapatites, the elimination of the latter by thermal treatment below 600° does not essentially modify the crystal structure But where pseudoapatites with a low Ca/P ratio are concerned, the liberation of water between 600° and 700° induces the formation of a β structure for pseudoapatites with a high Ca/P ratio the apatite lattice is stable

Another fundamental difference between apatites and pseudoapatites no doubt lies in the isotope exchange reaction

Considering the properties of calcium phosphates in relation to the theory of defect pseudoapatites, one is struck by this fact defect pseudoapatites fix calcium ions when immersed in a lime solution We previously thought with Posner that defects progressively filled up at this time but this view is not correct These calcium ions set on some surface (they can exchange with ^{45}Ca) and the defects are still present in the structure Saturation in the depth of the lattice seems to require considerable energy

Indeed, high temperature calcination does not alter the chemical composition of the compounds, but the ability to exchange excess calcium is greatly reduced

As shown by Fig 6, the percentage exchange of bone salts decreases progressively if they have been ignited at 300°, 400°, 500°, 600° and 700° C and reaches its lowest value at 700° C As an explanation of this fact, we postulate that calcium originally fixed on the surface moves under the influence of calcination to enter the defects, ousting the electropositive element which first occupied them This electropositive

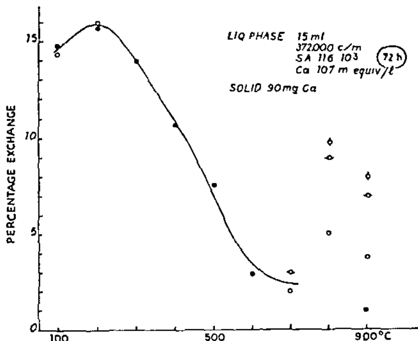


Fig 6 Percentage exchange of bone salts heated at different temperatures ranging from 100 to 900 C

element is probably an H_3O^+ group in mineralized bone and some organic radical in total bone For temperatures higher than 700° C (Fig 6) the results become very inconsistent from one experiment to another

Total bone and mineralized bone

The use of radiocalcium seems to be the best method for studying the behaviour of bone salts included in the organic

matrix Here are a few facts we have collected so far First, it takes much more time for ^{45}Ca to fix on total bone than on isolated bone salts In the latter case, equilibrium is reached

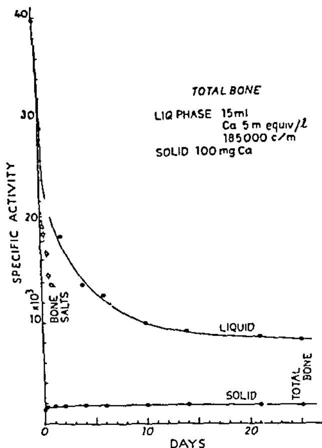


FIG 7 Rate of introduction of ^{45}Ca into total cow bone (a sample of 100 mg for each point) For comparison the curve obtained for bone salts (o o) is shown on the graph

in about 20 hours (if no recrystallization takes place) For total cow bone (Fig 7) 20 days are needed for the ^{45}Ca uptake to slow down Such differences could be interpreted as being due to the influence of organic matter which hampers diffusion Moreover, this exchange curve is absolutely regular and does not show any rapid phase of surface exchange

In the case of total cow bone, the percentage exchange at equilibrium reaches higher values (> 20 per cent) than for glycol ashed bone, even for the lowest $CaCl_2$ concentration (5 m equiv /l) in the liquid phase Furthermore, it seems to be less sensitive to the ionic strength ($CaCl_2$) of the liquid phase Slightly different results are obtained for embryonic bone For instance, chicken embryo bones exchange 13 per cent of their calcium (100 hours of contact) when immersed in a ^{45}Ca solution with very dilute $CaCl_2$ (0 32 m equiv /l), but more than 50 per cent is exchanged when the concentration in CaCl is 16 m equiv /l The rate of the exchange reaction is much higher than for cow bone

The conception of recrystallization as the main mechanism of calcium exchange in total bone *in vitro* seems to be questionable because of the high reversibility of this process These results obtained with total bone show that it behaves in a different manner from glycol ashed bone

Whence do these differences proceed? Some binding between bone salts and the organic part is probably involved We expressed this hypothesis in 1943, since then, it has been supported by *Neuman, Toribara and Mulryan (1952)* Results from Cagliotı, Ascenzi and Scrocco (1955) certainly corroborate these views Making use of infrared spectroscopy, these authors observed a binding between the SO_4 groups of the organic part and the PO_4 groups of the mineral part of bone

In conclusion, we may sum up some experimental facts related to bone mineral structure and our interpretation of them

Whatever the relative concentrations of phosphoric acid and lime mixed to obtain a phosphate precipitate this has always a defect pseudoapatite structure with a Ca/P weight ratio ranging from 1 72 to 2 26

A Ca/P ratio higher than 1 94 characteristic of TCPH, results from chemisorption of excess calcium This calcium is exchangeable in glycol ashed bone and synthetic phosphates Ignition causes the pseudoapatites to transform into apatites

with loss of water, loss of adsorption ability with regard to water and mineral ions, and loss of excess calcium exchange ability

We think that during ignition the chemisorbed excess calcium moves into the defect positions of the lattice The mechanism of ^{45}Ca incorporation in glycol ashed bone depends on the ionic strength of the liquid phase The results obtained with total bone show that it behaves in a different manner from glycol ashed bone The binding between the organic and mineral parts of bone may be responsible for this fact

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Bélanger If there is some Ca which is not actually bound with the crystals is it possible to envisage it as bound to some other portion of the bone, maybe to the organic fraction of bone and still exchangeable?

Dallemagne Yes it is possible That is a very difficult question At present, I imagine that in bone the defect position of the lattice can be completed by some radicals of the organic matrix for instance NH_2 or SH groups Probably this bond between the organic and the mineral fraction maintains the defect structure

Armstrong The concept of recrystallization of the bone mineral has always been a very interesting one but you now indicate that this process may not be so important as we had previously supposed it to be, particularly with regard to glycol ash and to mineralogical hydroxyapatite I should think that one important factor is the size of the crystal which very largely determines the surface area exposed to the solution Certainly, one of the limiting factors would be the boundary between the discontinuous and continuous phases It is quite possible that the glycol ash crystals are huge in comparison to the crystals of the ignited bone and therefore have a limited surface area as compared to those of untreated bone

Dallemagne When bone has been ignited at 500° C the curve of equilibrium is parallel to the curve of equilibrium at 100° C You have the same rate of exchange but not the same extent

Engström Following on Prof Armstrong's remark, I think the ignited bone and the mineral hydroxyapatite have about the same particle size judging from the diffraction patterns that is a particle size of about a couple of thousand Å What is your crystallographic evidence for this defect? There must be tremendous defects

Dallemagne We have no crystallographic evidence for that

Engström One usually talks about defect crystals (very small defects) but your results indicate a defect of the order of 10 per cent

Dallemagne It is impossible to obtain crystallographic information about that because the X ray diffraction lines are too broad It is impossible to make an accurate comparison between the X ray diffraction line of Arnold's compound (Ca/P ratio = 1 72) and the tricalcium phosphate line

Engstrom Yes but consider the profiles of the lines

Dallemagne We find some proof of the defect structure in the chemical properties of the mineral compound

Engstrom Do they differ significantly enough in refractive index?

Dallemagne I think the information given by refractive index is not very interesting because it depends largely on the water content

Engström This is certainly a defect one has seldom met before in crystals of this type it is very large

Dallemagne Yes the defects can involve at least two Ca atoms/6 P atoms These defects are probably filled by something H^+ ions or H_3O^+

Engström I think the whole secret of this lies in the very small particles Most calcium phosphates give hydroxyapatite when you store them in water or boil them in water everything you buy called

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DISCUSSION

de Bernard Prof Dallemagne what do you mean by ' exchange site of the inorganic particle?

Dallemagne We do not exactly know what is the situation of the exchangeable Ca atoms of bone mineral If only surface Ca atoms were involved in this exchange would be nearly instantaneous According to our experiments and those of Falkenheim and co workers this exchange is not rapid enough to be dependent only on surface phenomena Two explanations can be presented to explain this delay first a diffusion process may precede the exchange reaction secondly a physicochemical factor such as the ionic concentration of the liquid phase containing ^{45}Ca may slow down the exchange reaction

de Bernard Do you think that the content of water in the bone is concerned with the possibility of exchange of Ca?

Dallemagne Probably Neuman published some evidence in support of this fact the hydration layer seems to be very important in the mech anism of the exchange reaction According to this author when the hydration layer is lost by heating exchangeability is reduced

Lacroix Is it absurd to imagine that the excess Ca is located in the newly deposited layers and not in the fully calcified layers? It might be possible to check the idea since newly deposited layers are more numerous in the metaphy is than in the epiphysis

Dallemagne It would be very difficult to support such an explanation Indeed if the totality of the excess Ca were located only in the newly formed layers its concentration would exceed the surface fixation possi bilities of the crystals It would be interesting to know if in the newest and the oldest crystals the situation of the excess Ca atoms is the same one could imagine for instance that in the new crystals the excess Ca is located on the surface On the other hand in the oldest crystals it could be located in deeper positions in the lattice We observed *in vitro* that such a migration of Ca atoms is possible and we think that this represents the first phase of recrystallization When calcium phosphate precipitates very quickly we obtain a defect structure with Ca atoms (the excess Ca located on the surface) But when precipitation occurs slowly the structure is more complete and the defects are filled by the excess Ca This must only be considered as a working hypothesis and we are now engaged in checking its reality

do you believe is retained in the bones or in the body in a dry form and do you also know that chloride may be retained in the body in a dry form?

Dallemagne Bone contains a very small quantity of chloride, but the quantity of Na is rather high I think 0 7 mg per cent

Blaxter It is extremely difficult to determine the Na in bone by ordinary chemical methods because of interference by the large quantity of Ca and P present, but in collaboration with the Sheffield workers we have determined the Na in bone by isotope dilution and it comes out at about 400-500 mg /100 g We have found in analysis of the soft tissues of cattle for Na and K that we can predict their water content from their Na and K content with bone the Na is in excess relative to water content

Dallemagne I think that bones contain more than half the Na content of the body

Nordin There is some published work on this it came out at just under half the total body Na

tricalcium phosphate is hydroxyapatite I think it is the only naturally stable calcium phosphate

Blaxter When one comes to surface exchange one has Ca exchanging not only with other Ca ions but presumably for Mg possibly for K and for other ions Well those ions differ markedly in ionic radii and if recrystallization takes place as it presumably does how do they enter into the crystal without causing distortion?

Dallemagne We have some information about the Mg position the greater part of bone Mg seems to be adsorbed on the surface of phosphate crystals

Blaxter There is a very large difference

Dallemagne It would be interesting to try to fill the defects for instance with Ba ions or Mg ions

Blaxter Na will exchange Na has got an ionic radius which is about the same as that of Ca

Dallemagne But when bone mineral is ignited and is immersed in water the totality of the Na goes into solution Probably it is completely absorbed

Engstrom The ionic radii of Sr and Ca however are about the same so I would think that one could replace Ca by Sr You can build up intermediates in the dry way i e by melting compounds together If you try to precipitate them together you do not get intermediates either strontium apatite or calcium apatite is formed

Dallemagne Next year we will try to introduce some other ions such as Mg or Sr into the phosphate lattice

Engstrom If you have Mg or certain other ions present when you do the precipitate this seems to stop the development of the crystallites

Armstrong There is not very much Mg in bone and patients receiving Mg show positive Mg balances without change in the Ca balance The supposition is that Mg is not simply replacing Ca in bone

Meyer In non mineralized connective tissue there is quite a considerable amount of Na and other minerals Is it not possible that the site of these extra minerals what you call the deficit or the excess depending on which side you look is not located at all at the same site where you have pseudohydroxyapatite? It is located in the amorphous organic part of the ground substance in the bone and therefore this really has nothing to do with the very regularly arranged crystal structure In other words this consists of inorganic non crystalline salts I think it would be quite interesting to do the same type of analysis in tendon and see how this would behave in all your systems

Engstrom In doing these isotope labelling experiments we have not yet been able to get any isotope which does not localize to these lowly mineralized structures both *in vivo* and *in vitro* whether Ca Sr PO_4 SO_4 or Na

Fanconi We demonstrated 10 years ago in nephrosis that we must have some of the Na and also some of the chloride retained in the body in a dry form Now we hear that Na is retained to a great extent in the bones in a dry form We were of the opinion that the collagen fibrils are the site of chloride in a dry form and I ask as a clinician how much Na

of calcium It is obvious that the combined illustration expresses the sequence of events

Histological observations have been made on ground sections decalcified in a solution of sequestrene at pH 7

The inner zone, which we shall call the preosseous layer, is orthochromatic with toluidine blue, it is PAS and Bauer positive, it takes selectively Alcian blue and ruthenium red and it is made metachromatic by the action of sulphuric and chromic acids, methylene blue extinction occurs at pH 4 The peripheral zone is metachromatic and methylene blue extinction occurs at pH 5 6 Most of these features are those of mucopolysaccharides

In the fully deposited osteon, metachromasia is inversely proportional to the content of calcium

This histological study (Vincent, 1954) is a development of previous remarks made from a comparison between the microradiographic and the histological pattern of undecalcified ground sections (Cohen and Lacroix, 1953)

In a short note on bone growth in rabbits and rats, Arnold and Jee (1954*a*) have reported some observations which are in good agreement with the preceding description

The main point seems to be that a new layer of bone changes its orthochromasia into metachromasia when it begins to manifest a strong affinity for calcium Related observations have been made by Levine and co workers (1949)

Let us go one step further and compare microradiographs with autoradiographs With the relevant histological observations in mind, we are in a better position, and the microradiographic reference will, from now on, mean much more to us than a mere map of calcium deposits

The first isotope that we used was ^{45}Ca (Lacroix, 1952,1953, 1954) Seven days after administration to an adult dog, the areas of radioactivity (Fig 2A) when superimposed on the microradiograph (Fig 2B), are found in the layers which begin to calcify With adequate methods a very faint radioactivity may be detected in the osteons which are completely built but not yet fully calcified The distribution *in vivo* of

THE HISTOLOGICAL REMODELLING OF ADULT BONE

AN AUTORADIOGRAPHIC STUDY

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THE main purpose of this study has been to take advantage of isotope techniques to obtain a better knowledge of the histological remodelling of the adult bone Furthermore, it was hoped that the research would provide some information on bone matrix

Compact bone in the dog

Microradiography has certainly been one of the most useful tools in recent studies on compact bone (Engstrom, 1949, Amprino, 1952, Amprino and Engstrom, 1952) The pictures provided by this method are now familiar to all of us They prove that calcification of compact bone occurs in two stages At first, about three quarters of what will be the final load of calcium is stored by the osteon (Haversian system) while it is built up Then, after osteogenesis has ceased, calcification of the osteon is slowly completed

The microradiograph does not tell the whole story of the formation of an osteon In fact, calcification begins in a layer which already existed some time before The information is obtained by comparing the microradiograph with the histological picture (Fig 1) Two concentric zones may be observed in an osteon which is being laid down The inner zone is seen only with the microscope (long arrow of Fig 1B) Since it does not appear on the microradiograph (Fig 1A) its calcium content is nil or very low The peripheral zone (short arrow of Fig 1B) corresponds to a rather heavy fixation

Fig 1 An example of correlation between microradiography (A) and histology (B) in the study of compact bone During the process of deposition an osteon is made up of an inner zone (long arrow) the preosseous layer which does not appear on the microradiograph because it is uncalcified and of a peripheral zone (short arrow) which calcifies The inner zone is orthochromatic and the peripheral zone is metachromatic (× 107)

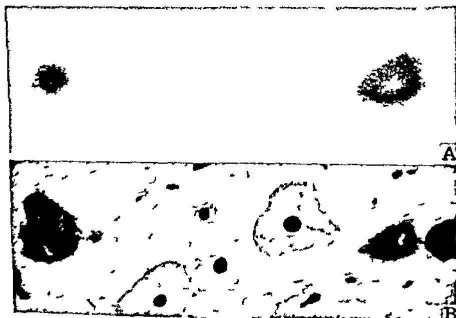


Fig 2 Distribution of ^{45}Ca in compact bone seven days after administration as shown by an illustration combining the autoradiograph (A) and the corresponding microradiograph (B) The radioactivity belongs to the layers which begin to calcify (× 85)

[facing page 38

^{45}Ca, therefore, entirely confirms what the microradiograph indicates about the dynamics of calcification very fast at first, very slow afterwards

Analogous autoradiographs have been recorded with ^{3} P (Engfeldt, Engstrom and Zetterstrom, 1952) and with ^{90}Sr (Jowsey, Owen and Vaughan, 1953, Engfeldt *et al*, 1954)

Autoradiographs obtained seven days after administration of ^{35}S are similar at first sight to those produced by ^{45}Ca, but they are not absolutely identical Here, the areas of radio activity (Fig 3A) are surrounded by the layers which begin to calcify (Fig 3B), instead of corresponding exactly to them, in other words, they belong to the preosseous layers, the exist *ence of which is proved by the observations exemplified by* Fig 1

The life of adult bone implies therefore a metabolism of sulphur which precedes that of calcium Whether the former directs the latter is open to speculation (*cf* on this particular point the suggestions of Neuman, Boyd and Feldman, 1952)

From the foregoing, some conclusions have been drawn pertaining to the value of radioactivity measurements

If we compare the specific activities of samples of bones, free of all traces of marrow, seven days after ^{35}S administra tion, the figures will give directly a relative estimation of the osteogenesis going on in the samples

Similar measurements with ^{45}Ca amount to comparing the calcification which occurs in the samples Calcification follow ing in the wake of osteogenesis, we may deem that the measure ments will also mean indirectly a relative evaluation of osteogenesis

The short term localization of ^{35}S in the preosseous layer, which is orthochromatic is at variance with the histological localization found in other tissues All the observations recorded up to now (*cf* Bostrom, 1953) indicate a close relation ship between ^{35}S incorporation and metachromasia We shall see presently that, in a way the relationship holds true for bone also because the radioactive layer will acquire metachromatic properties some days later



Fig 5 Autoradiograph (A) of compact bone eight weeks after ^{35}S administration with corresponding microradiograph (B) Some osteons (e g those indicated by arrows) may have practically the same calcium content although they are not contemporary (× 93)

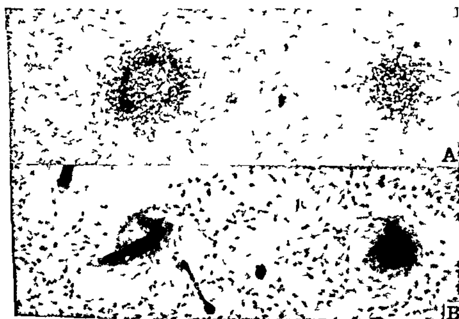


Fig 6 Autoradiograph (A) of compact bone eighteen weeks after ^{35}S administration Even at this later stage some radioactive osteons are not yet fully calcified (× 115)

Fig 3 Distribution of ^{35}S in compact bone seven days after administration The pattern of radioactivity (A) is not identical with that of Fig 2 A The radioactivity belongs now to a structure which is not shown by the microradiograph (B) most likely to the preosseous layer the existence of which is proved by Fig 1 (× 85)

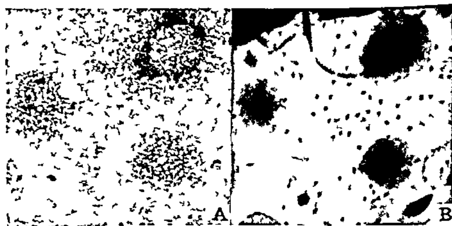


Fig 4 Autoradiograph (A) of compact bone six weeks after ^{35}S administration The radioactive layers i e the layers labelled at the time of injection are now at the periphery of osteons which according to their calcium content (B) have just been deposited (× 105)

Six weeks after ^{35}S administration (Fig 4) the correlation is easy to read, if we admit that the initial distribution of the isotope has not been substantially altered, that, in other words, the radioactive layers are still those which were labelled at the time of injection Fig 4 shows that the radioactivity is now at the periphery of osteons, which, according to their low calcium content, have just been built The case seems fairly typical and we may say that the deposition of an osteon takes about six weeks in the adult dog This is only a rough estimation because the process of remodelling may sometimes stop completely for a while

Working with ^{45}Ca, Arnold and Jee (1953, 1954*b*) and Jee and Arnold (1954) indicate that the deposition of an osteon is generally a matter of more than three weeks in the growing rabbit

Eight weeks after injection of the isotope (Fig 5), we begin to realize that some osteons (*e g* those indicated by arrows) may have reached practically the same level of calcification although, according to the autoradiograph, they are not contemporary This means that the completion of calcification is so slow that we cannot rely on the microradiograph to ascertain the relative age of two fully deposited osteons

At the eighteen week stage, some of the radioactive osteons are almost fully calcified, but some others (Fig 6) do not show much progress from the preceding stages

Since Figs 4–6 prove (by comparison with Fig 1) that the radioactivity belongs now to metachromatic layers, it appears that a relationship between ^{35}S incorporation and metachromasia exists indeed in adult bone but is more dynamic than static This is an additional indication in favour of a basic function of mucopolysaccharides in bone metabolism

One may surmise that an adult dog given ^{35}S at regular and short intervals during six weeks and killed three weeks afterwards, would have most of the bone radioactivity in metachromatic regions The biochemist who could extract from the bones of such a dog a radioactive identifiable

Fig 7 Autoradiograph (A) of metaphyseal trabeculae seven days after ^{45}Ca administration to an adult dog The corresponding microradiograph (B) shows that the radioactivity belongs to the layers which begin to calcify (× 114)

Fig 8 Autoradiograph of a longitudinal section of the upper tibial extremity seven days after ^{45}Ca administration to an adult dog The radioactive layers are located almost exclusively in the metaphysis (× 4 7)

Knowing now the significance of ^{35}S and ^{45}Ca distribution in bone, we may safely state that the histological remodelling is more active in the metaphysis than in the epiphysis

The higher specific activity exhibited by whole extremities, as compared with the diaphysis, is therefore an average which does not take into account the difference which we have just pointed out

But the ratio between epiphysis and metaphysis put together, on the one hand, and diaphysis, on the other hand, rough as it is, remains nevertheless useful Since, on the whole, the remodelling is more active in the extremities than in the diaphysis, it follows that, in the long run, the specific activity of the extremities should fall at a faster rate than that of the diaphysis

Data are still meagre on this question Lontie (1953) has observed that in the adult rabbit injected with ^{45}Ca, the proportional distribution of specific activities in the long bones does not change significantly in a period of about two hundred days It seems therefore that the histological remodelling, *i e* the complete destruction of old bone and its replacement by new bone, is very slow indeed

The subject deserves further study in relation to current theories on the rôle of the skeleton in calcium homeostasis It is now technically possible to figure out the amount of bone which is morphologically renewed in a given time A comparison with the turnover of ^{45}Ca would be of much significance and might throw some light on the way in which calcium is removed from the skeleton

It may be fitting to recall here that the bone marrow shows some histological individuality in the epiphysis and in the metaphysis and that both regions derive their main blood supply from distinct sources (Trueta and Harrison, 1953 , Judet *et al* 1954)

These considerations are admittedly hampered by the fact that all available information comes from animals of different species and sizes They apply of course to the adult only and not to the growing skeleton where the situation is quite

compound would provide us with valuable new data on bone matrix

It is encouraging to note that along the same line of work it has been found possible to extract from the young epiphysis (Dziewiatkowski, 1951), from the costal cartilage (Boström, 1952) and from the skin (Bostrom and Gardell, 1953), a radioactive mucopolysaccharide, in these cases chondroitin sulphuric acid

Engfeldt and Hjertquist (1954) have already shown that ^{35}S is incorporated in the organic portion of bone and also in the inorganic portion Belanger (1954) has suggested a similar distribution Furthermore the presence of mucopolysacchar ides in bone has been recognized for many years (*cf* Glegg and Eidinger, 1955)

Cancellous bone in the dog

In research on cancellous bone, the main problem was to prepare sections which would be even and thin enough to be microradiographed The difficulty has been solved by em bedding the specimens in methylmethacrylate and then by sawing slices which were ground like compact bone

The microradiograph shows that the trabeculae of cancell ous bone are made up of layers with varying calcium content The general design may be likened to that of compact bone

The distribution of ^{45}Ca (Fig 7) and of ^{35}S seems to obey the same laws in both types of bone tissue

It seems also that the deposition of new cancellous bone implies two stages the formation of a preosseous, uncalcified orthochromatic layer, and the sudden appearance in this layer of metachromatic properties linked with a strong affinity for calcium

As a matter of fact, all these features are much more easily observed in the metaphysis than in the epiphysis The difference is such that sometimes the radioactivity is found almost exclusively in the metaphysis, as is shown by Fig 8, a ^{4} Ca autoradiograph of the upper extremity of the tibia Comparable aspects have been observed with ^{35}S

first stage of which is a radiographic translucency of the metaphysis

Acknowledgements

This presentation is based chiefly on unpublished research carried out in the author's laboratory by J Vincent who will give a full account of his work in a monograph due to appear later *

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* NOTE ADDED IN PROOF this work has since been published (Vincent 1955)

different (*cf*, on this latter point, Lacroix, 1951, Bauer, Carlsson and Lindquist, 1955)

Human bone

In adult human bone, the formation of a preosseous layer is less easily observed than in the dog On the other hand, the relationship between the metachromatic properties of an osteon at the successive stages of its formation and its affinity for calcium is more obvious

Incidentally, we may confirm the statement of Engstrom and Engfeldt (1953) that, in the osteon itself, the calcium content is alternatively low and high from one lamella to the other The differences fade out when the osteon is fully calcified The observation seems to meet those made with the electron microscope by Rouiller and co workers (1952), but it must be admitted that the bridge between both sets of facts is still lacking

Generally speaking, comparison between man and dog indicates that the dog is a good experimental animal for research aiming at results which may be valid also for man

Remarks

We hope that our survey will have shown that compact bone has several advantages of its own in the study of problems of bone physiology and pathology The material lends itself easily to correlated microradiographic, autoradiographic and histological examinations It shows in the same field the expression of stages where the metabolism of various elements may be visualized at the microscopic level The succession of events being slow, it presents opportunities to dissociate from each other processes which possibly overlap elsewhere

The interest of cancellous bone is different and lies in the fact that the adult keeps something of the structural differences prevailing during growth between epiphysis and metaphysis The observation might lead to an understanding of the acute post traumatic osteoporosis of the adult, the

Meyer How do you distinguish between inorganic and organic sulphate?

Engström The first fraction came out with simple water extraction after which there was still some radioactivity left in the bone

Meyer So one can only talk about insoluble sulphate, whether this is organic or inorganic you cannot say

Engström No of course We tried to extract this "organic" fraction but the amounts were too small to permit estimation of the specific activities

Amprino I would like to point out to Prof Engstrom that it is not certain whether the radioactive broad band underlying the epiphyseal plate is only due to the sulphate fixed to the bone tissue because there is at that very level a lot of calcified cartilage which is the remnant of the lower part of the epiphyseal plate which has been partially resorbed So we have there both bone and cartilage and we know that cartilage fixes a large amount of radioactive S We should therefore, be quite cautious when interpreting the autoradiographic aspect of the epiphyseal plate region

Rodicel Prof Lacroix your Fig 1 showed the sequence which leads up to calcification a very similar picture is obtained in healing wounds skin or tendon wounds where you also have this sequence of course without Ca deposition I would suggest that the true causes of calcification or of eventual deposition of Ca, are not the structures which are revealed by sulphate uptake or metachromasia or PAS staining because otherwise why should the skin or tendon not calcify as well?

Lacroix Some data in the literature strongly suggest that mucopolysaccharides play a rôle in calcification

Armstrong You have shown us what I think is very good evidence for the time required for the formation of calcification of an osteon Is it possible to indicate how long an osteon endures?

Lacroix The question is now being studied It is a problem of great importance, because what we need most at the present time is a comparison between the rate of the histological renewing and the biological half life of ^{45}Ca Up to now the biological half life of ^{45}Ca has been studied in the so called adult rat which does not stop growing or in the almost fully grown animal as far as I know it has not been satisfactorily recorded in really adult bone

Armstrong One would assume that the number of osteons in an adult ought to be constant or nearly constant This would then require that the number formed should equal the number removed

Lacroix Yes roughly It would perhaps be more accurate to state that considering a long period of time in a young adult in perfect health the total amount of newly formed lamellae equals that of removed lamellae

Armstrong Can you see anywhere except in the endosteum or periosteum any evidence of the resorption of a radioactive osteon?

Lacroix No It would take, I think several months before we would have a chance of seeing a radioactive osteon being eaten up We may perhaps infer from what happens in radium poisoning in man There are

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DISCUSSION

Fanconi I have X rays of bones from children with lead poisoning These X rays present a lot of lead just in the metaphysis as you said that is the most active part of the bone and not the epiphysis or the diaphysis

Lacroix Your remark proves that lead deposition is linked with osteogenesis This suggests the technical possibility of using lead instead of radioactive isotopes as a tool in the study of the histological remodelling of adult bone

Follis We have studied the histological appearance of lead in the bones of children At the Johns Hopkins Hospital we had a series of about 60 cases The dense shadow as Dr Park showed many years ago in lead poisoning in children is due in large part to the persistence of excessive amounts of calcified cartilaginous matrix encased in large amounts of bone The defect there apparently is a poisoning of the destructive mechanisms so that the calcified and probably plumbified matrix remains, the bone encasing it remains and both give rise to the bright line one sees in the X ray

Engstrom We did some experiments with radiosulphate in dogs and radioautographs from longitudinal sections of the long bones showed as was known before a heavy deposition in cartilage but also in the bone tissue itself If you take spongious bone and powder it and compare its radiosulphate activity with powder from compact bone the total radioactivity is about twice as high in the spongious bone Then we tried to separate two fractions containing radiosulphate and we found that about one half of the radioactivity goes to the inorganic portion and about half of it to the organic You can take a bone section non radioactive and incubate it *in vitro* and you obtain uptake in the inorganic fraction of bone and nothing in the organic This means that a fraction of the $^{35}SO_4$ goes to the inorganic portion and is probably surface bound by exchange

FIBROGENESIS AND THE FORMATION OF MATRIX IN DEVELOPING BONE

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Introduction

IN an effort to obtain a better understanding of the basic structure and early development of bone an investigation has been made of the various components of osteogenic cells and an attempt has been made to relate the function of these cells to the morphogenesis of the organic intercellular material of bone in the fowl embryo This work falls into three sections firstly an optical study of living bone forming tissue, secondly, a cytochemical investigation of the components of osteoblasts correlated with histochemical data, and thirdly, electron microscope observations on cell structure and the formation of bone matrix with some preliminary analysis by means of electron diffraction

Experimental Observations

1 Optical Study of Living Bone-forming Tissue

Living tissue cultures of long bone and frontal bone of the fowl embryo have been grown in a fibrin free liquid medium and viewed either by phase contrast or interference microscopy The general growth seen to take place in the cultures

two papers on the question one (Hoecker F E and Roofe P G (1951) *Radiology* 56 89) showing that 7 years after radium poisoning the pictures of radioactive osteons are still complete rings and the other (Looney W B, and Woodruff L A (1953) *Arch Path (Lab Med)* 56, 1) in which there are instances of radioactive osteons partially destroyed 22 years after radium incorporation However the meaning of these facts remains uncertain because the poisoned bone tissue is certainly abnormal

Lisco I noticed from your pictures that the amount of radioactivity that you have used in order to demonstrate the activity by radioautographs was fairly considerable and the question arises as to whether or not such an amount of radioactivity could have interfered with the formation of such an osteon?

Lacroix The fact that radioactivity may by itself impair the deposition of the osteon remains open to question and it is one of the numerous objections which may be raised against that type of work That is why I wish that something other than radioactivity could be used for instance lead or vital staining

Lisco In other words we may have to revise this figure of six weeks for the formation of the osteon a few years hence Furthermore I wonder whether you have given consideration to the possibility that marked species differences might exist for the time that is required for the growth of an osteon to be complete

Lacroix I completely agree I was careful to point out that it was a rough estimation

Lisco I noticed that

Follis If you were to study the difference which you showed between the metaphyseal and epiphyseal trabeculae at various stages while the cartilage was still there would you again find this difference?

Lacroix Yes we may sum up by saying that the adult at least the young adult keeps something of the differences which prevail during growth


2


1


3


4

FIGS 1–4

has been recorded by time lapse cine microphotography, it is possible by obervations of this kind to follow the behaviour of the cells and the development of the intercellular material

After incubation of the cultures for twenty four hours a thin halo of migrating osteoblasts is seen to surround the sharply cut edge of the explanted tissue, by thirty six hours a peripheral zone of outwandering osteoblasts has developed and forms a typical network with cell processes The immediately striking feature of the migrating osteoblasts is their cytoplasmic content of a large number of ovoid bodies of low optical density similar in appearance to small vacuoles As the cultures age these bodies increase in density and take the form of granules of 0 7 μ to 2 μ in diameter (Fig 1) They are generally clustered around the nucleus, but also spread into the cell processes where they show considerable mobility The granules may easily be distinguished from the filamentous mitochondria typical of such cells in culture They are composed of a less refractile material than the smaller lipid globules that are also present Intact granules have not been seen to be extruded from the cells

With the interferometer microscope the variations in mass per unit area in the cell show up as variations in the photographic density of the image when recorded in monochromatic light (Davies *et al*, 1954) if white light and colour film are used these variations are seen as changes of colour When living cultures are observed by this latter method colour

PLATE I

FIG 1 Phase contrast photomicrograph of part of an osteoblast growing from a living culture of frontal bone Cytoplasmic granules are clustered above the nucleus filamentous mitochondria are also visible in the cytoplasm (× 1 500)

FIG 2 Photomicrograph by phase contrast of the peripheral growth in a living culture of frontal bone from an 11 day fowl embryo after 48 hours in culture The cytoplasmic regions are packed with granules some of which may be seen in the cell processes (× 270)

FIG 3 The same field (slightly rotated) after 65 hours growth There is now an orientated condensation of osteoblasts (× 270)

FIG 4 The same field after 90 hours growth Osteocytes are now enclosed by extracellular material which has begun to ossify (× 270)









FIGS 1–4

changes are particularly noticeable at the ends of fine cyto plasmic processes, the projected areas of which are found, by measurement, to remain constant Hence the mass of the process must increase and diminish in alternating sequence It seems improbable, however, that such variations in mass are due to changes in the thickness of the cytoplasm for this is unlikely to occur without some alterations in the projected area Rather, the changes would seem to be due to variations in the concentration of material in the processes A quantitative estimation of the magnitude of these changes would require the use of monochromatic light and densitometry

The general development of the intercellular material may be studied when the same field of a tissue culture is kept under continual observation After migration of the peripheral zone of osteoblasts, the inner region of outgrowth shows signs of a condensation of cells and the cytoplasmic granules are well in evidence (Fig 2), after about sixty five hours' growth the cells become orientated particularly near the cell boundaries and granules are visible (Fig 3) Irregular regions develop between the cells, and the use of polarized light demonstrates the presence of form birefringent material in these areas This suggests that osteogenic fibres are being deposited It then becomes impossible to distinguish clearly between the cell surfaces and intercellular material for an opaque substance begins to cover the condensed mass of cells until it tends to mask the whole area Gradually, however, the intercellular regions enlarge until the individual cells become enclosed and immobilized by bone matrix (Fig 4) Some movement of granules can still be detected in the cells at this stage Calcification in such regions of the cultures can be demonstrated by silver staining When the metabolism of the cultures is inhibited by a lowering of the temperature to 27° C the granules are seen to decline in number and intercellular material does not develop Most of the cells however during this period do not show any of the signs usually associated with degeneration

These observations of the living osteogenic tissue *in vitro*

A new type of bulk tissue culture has been devised for use with biochemical experiments Suspensions of osteoblasts obtained directly from the frontal bones of fowl embryos are grown in a fluid fibrin free medium The method of preparation of the cell suspension for culture is a modification of the technique of Moscona (1952) The cells are obtained from the newly formed bone rudiments by means of careful dissection from the embryos followed by incubation at 38 5° C in 0 01 per cent crystalline trypsin in calcium and magnesium free Tyrode solution The cells are then washed free of trypsin and separated from debris by centrifugation with normal Tyrode and filtration through 200 mesh platinum wire Such cell preparations have been found to be essentially free of characteristic collagen fibrils when inspected in the electron microscope The cells are suspended in the prepared culture medium and subsequently spread thinly over the flat upper surface of the culture chambers, each chamber is then sealed by means of an inverted watch glass and paraffin wax The cultures are incubated at 38 5° C, the viable cells quickly settle on the glass and increase in number by mitosis, if the cultures are harvested after a period of forty five hours it is found that there is about a six fold increase of material (by weight) By the use of the electron microscope it has been shown, however, that there is a negligible development of characteristic collagen fibrils at this stage The cytochemical properties of these cells after forty five hours' growth are essentially similar to those recorded in osteoblasts growing from explanted bone tissue Thus, this method of culture provides a sufficient number of osteogenic cells suitable and free of contamination with extracellular material for bio chemical experiments If the cultures are grown for longer periods however, intercellular substances develop in con siderable quantity

Cultures of osteoblasts grown in this way for periods up to forty five hours were incubated with $^{35}SO_4$ (given as inorganic sulphate) for three hours prior to harvesting After washing and drying, the cells were found to have taken up a significant

indicate strongly that the presence of the cytoplasmic granules may be correlated with the formation and development of the intercellular material

2a Cytochemical Properties of Osteoblasts *in vitro*

The cytochemical properties of osteoblasts in tissue culture have been studied extensively (Fitton Jackson, 1955), some observations by other workers have been confirmed and new data have been obtained

When neutral red is introduced into the cultures as a supravital stain the granules become pale orange, indicating that they must have a pH of about 7–7 5 When either toluidine blue or thionine is added separately to the living cultures the granules are seen to take up a red purple colouration which shows that they have metachromatic properties in the living state

After fixation of the cultures in, for instance, cold absolute methanol the granules may be stained by the use of the periodic acid Schiff (PAS) techniques of McManus (1946) or Hotchkiss (1948) This reaction can still be obtained after extraction of either lipid from the cells with a methanol chloroform mixture at 50° C for twelve hours, or after exposure to amylase for the removal of glycogen The presence of a free aldehyde group in the granules can be excluded since the Schiff reagent stains only after prior treatment with periodic acid It is generally accepted that $C_{(1)}$ and $C_{(2)}$ glycol groups are stained by application of the PAS technique and prevention of this reaction has been obtained after acetylation according to the method of McManus and Casson (1950) The granules are stained blue by the use of the iron absorption technique devised by Hale (1946) this colour is considered typical for acid polysaccharide

The cytochemical observations of positive reactions with the PAS and Hale techniques the presence of metachromasia and the property of a pH of about 7 taken together indicate that the granules contain at least in part, an amino polysaccharide

methyl green pyronin methods stain part of the cytoplasm of the osteoblasts an intensive blue or pink red respectively (Jacobson and Webb, 1952) and such basophilia in the cells was shown by them to demonstrate the presence of ribonucleo protein (RNP) The stained material consists of a mass of fine particles separated by clear areas However, the cyto plasmic granules do not take up either stain, nor are they destroyed by digestion with either ribonuclease or desoxy ribonuclease for periods sufficient to remove the known con tent of RNP or DNP from the cells The absorption curve obtained from ultraviolet microscopy of the tissue culture shows there is a specific absorption maximum occurring at about 280 mμ, for the plateau effect obtained between 265 mμ and 280 mμ would not be expected if this absorption spectrum was due entirely to light scatter Furthermore, the perinuclear region is found to give a strong positive reaction and parts of the rest of the cytoplasm a weaker reaction by the use of the Mazia technique (Mazia, Brewer and Alfert, 1953) for the demonstration of protein These facts indicate that, in addition to the nucleoprotein and protein of the cytoplasm, the granules contain some protein

In the cells growing in regions where intercellular material is developing the granules give an alkaline phosphatase reaction by the use of the Gomori Takamatsu technique as modified by Danielli (1953) and this may be correlated with the known phosphatase activity of fibrogenic tissue (Fell and Danielli, 1943, Gold and Gould, 1951) Application of the G Nadi reaction (Moog, 1943) with the necessary inhibitory controls indicates that cytochrome oxidase is associated with the granular region of the cell it has been found that this reaction decreases in intensity when the culture is in a low metabolic state produced by a decrease of temperature

2b Histochemistry of Developing Bone *in vivo*

The same basic results reported above have been demon strated clearly in osteoblasts taken carefully from the embryo and prepared as freshly teased out preparations The only

amount of isotopic sulphate The dried cells were then extracted for amino polysaccharides with a 10 per cent calcium chloride solution for three days at 3° C, the extracts were then partially purified and they showed a negligible radioactivity count The infrared absorption spectra of these extracts were obtained after preparing thin films of the specimens on silver chloride plates A double beam reflecting microscope (N A 0 8) was used in conjunction with a Grubb Parsons I R spectrometer with a sodium chloride prism The absorption spectrum of the cell extracts was almost exactly the same as that of a non sulphated hyaluronic acid, but differed very markedly from the control samples of chondroitin sulphate and heparin When osteoblast cultures were grown for up to five days (i e when considerable intercellular material had developed) and then treated as above, the absorption spectrum of the extracts shows evidence of sulphate, the spectra, however, were not identical with either of the spectra of the control samples of chondroitin sulphate or heparin Although the whole cultures have been found to take up radioactive sulphate, it has not been incorporated in the material extracted from the osteoblasts by means of calcium chloride

If cultures of osteoblasts grown from explants are digested by 0 1 per cent hyaluronidase, essentially according to the method of Lillie (1947) for more than eighteen hours at 38 5° C the granules can no longer be stained clearly by the PAS or the Hale techniques As Meyer and Rapport (1952) have defined hyaluronidase as an enzyme which depolymerizes or hydrolyses hyaluronic acid, the alteration in the staining capacity of the granules after digestion by the enzyme indicates the presence of hyaluronic acid

Thus from the cytochemical results the infrared data on cell extracts, and the effects of hyaluronidase digestion, it may be concluded that the intracytoplasmic granules of osteoblasts contain an amino polysaccharide possibly of a hyaluronic acid type

It has been confirmed that the May Grunwald Giemsa and



FIG 5 Section of frontal bone of a 13 day fowl embryo showing organization of the cytoplasm in osteoblasts Typical mitochondria (M) paired lamellae (L) and particulate structures (P S) are visible The central area of the micrograph may represent the Golgi apparatus Part of the cell surface (C S) and intercellular spaces (I S) are also well defined (×32 000)

detectable difference lies in the size of the granules, in such preparations they are not greater than 0 3 μ in size The amino polysaccharide containing granules can also be demonstrated in frozen dried preparations of the embryonic tissues sectioned at 10 μ These granules are probably homologous with the Schiff positive cytoplasmic granules described by Heller Steinberg (1951) as occurring in osteoblasts of young rat bone

The histochemical properties of developing bone *in vivo* have been investigated by numerous people (e g Bevelander and Johnson, 1950, Pritchard, 1952), these workers showed that alkaline phosphatase, metachromasia and Schiff positive material were present in the forming matrix of mammalian bone We have obtained identical results for avian long bone and avian membrane bone

3 Electron Microscope Observations on Thin Sections of the Fowl Embryo

The tissues used were fixed in 1 0 per cent osmium tetroxide in Tyrode solution held at pH 8 2 by additional sodium bicarbonate (Fitton Jackson, 1956) and they were subsequently embedded in methacrylate (Newman Borysko and Swerdlow 1949) The plastic was not removed from the prepared sections

(*a*) *The development of membrane bone* Membranous ossification occurs directly by condensation and differentiation of mesenchymal tissue and independently of cartilage formation By electron microscopy in thin sections of the frontal bone, the angulare and supra angulare of the mandible the osteoblasts are seen to be oval in shape with spherical nuclei lying to one side of the relatively large cytoplasmic regions Parts of the cell surfaces (represented by a dense line about 70 Å wide) of adjacent cells are intimately associated with each other, but irregular spaces also occur between the osteoblasts and may contain blood corpuscles and developing blood vessels

The cytoplasm of the osteoblasts contains a system of

apparently curved, paired lamellae each about 70 Å in width and with a varying distance between each member of a pair (Fig 5) The internal surfaces of the lamellae pairs as seen in section are smooth, but the external surfaces are associated with fine particles about 100 Å wide, the space between each member of a pair is made up of very fine amorphous material of quite low electron density, but which is usually higher than that of the surrounding cytoplasmic matrix Some very fine dense particles of less than 40 Å are also seen to form separate particulate structures rather similar in outline to the image of the lamellae pairs This suggests that they may be a develop ing form of such components Other fine particles of about the same size and density are also scattered at random in the cytoplasmic matrix

Larger particles have been observed and are composed of an outer membrane confining a substance of medium density They are often found in association with large bodies which are possibly of a vacuolar nature, these are seen as irregular shapes of low density and each is delimited by a double membranous structure depicted by two lines about 40 Å in width and separated from each other by a distance of about 100 Å It is possible that the particles and vacuolar like bodies when taken together may represent the Golgi apparatus in the cell, for this region is closely similar in structure to the Golgi apparatus as described by Sjostrand and Hanzon (1954*a*) in the exocrine cells of the pancreas

Numerous ovoids with cristae or internal membranes are also present in the cytoplasm, generally such structures are taken to represent transverse sections of mitochondria as seen by electron microscopy (cf Palade 1952, Sjostrand and Hanzon, 1954*b*) However, in view of the evidence presented

PLATE II

FIG 6 In the top of the micrograph part of the cytoplasm of an osteoblast in frontal bone may be seen and just below there is an opaque granular mass which has formed in the intercellular region Collagen fibres are also visible the small dense particles may represent salt deposits (× 15 000)

FIG 7 A section of the shaft of the metatarsus of a 16 day fowl embryo shows part of an osteocyte surrounded by intercellular material (× 15 000)

Figs 6–7



Fig 8 Sections of parts of two osteoblasts from the periosteum of a 16 day fowl embryo showing collagen fibres lying in close association with the cell surfaces Periodic structure of the fibrils can be clearly distinguished while in one part an opaque granular mass indicates a calcification region (× 17 500)

[*facing page* 56

above (i e cytochemical) it is suggested that some at least of these ovoids are identical with the cytoplasmic granules

As stated above, at the beginning of differentiation of bone the cells are usually seen to be in contact with each other, but as the bone develops closely packed intercellular spaces occur which become partially composed of disorientated fibrils of varying diameter Some of these show the characteristic periodicity of about 640 Å of collagen fibrils in bone, while others, of diameter of not greater than 200 Å show no periodicity The cytoplasm of some of the osteoblasts is intimately related to the fibrils, for in places no cell surface can be distinguished, this suggests that the organic material is being formed in association with the cytoplasm

A substance of low electron density is also present in the intercellular material, where collagen fibrils have been cut transversely this substance is seen to invest each individual fibril The intercellular regions gradually enlarge and become filled with fibrils which form small bundles Electron dense particles are scattered at random throughout the area and may represent bone salts Granular material then gradually masks the collagen fibrils and enlarges to form opaque regions which may be identified with the first stage of calcification of the bone matrix (Fig 6)

(*b*) *Development of the organic matrix of periosteal bone and the first stage of calcification* When thin sections of the tarso metatarsus of five day embryos are viewed in the electron microscope the cells of the layer surrounding the cartilage are seen to have elongated nuclei, enclosed by a thin layer of cytoplasm These cells then differentiate into the outer fibro blastic and the inner osteoblastic layers of the periosteum The structure of the osteoblasts is essentially similar to that already described The innermost layer of the osteoblasts becomes separated from the adjacent cartilage by a narrow strip of material differing in composition from that of the cartilage matrix In the early stages it is made up of dis orientated fine fibrils less than 200 Å in diameter, and of collagen fibrils with a periodicity of about 640 Å with varying

generally considered that the mineral content of bone and teeth belong to this class of apatite (e g Trautz, 1955)] It would thus appear that apatite mineral is formed extremely early in the developmental process, whether such formation takes place in stages, or directly from the ions present in solution cannot be deduced from the present experiments

Discussion

Osteoblasts have been shown to contain cytoplasmic granules which are present mainly when intercellular material is about to be, or is being, deposited, it is therefore a reasonable assumption that they are concerned with its production As no extrusion of the intact granules has been observed, however, it is probable that a different mechanism for the transfer of their products from the cells must exist The indication that there may be variations in concentration of material in cytoplasmic processes suggests that cell products could diffuse from such parts of the cells, these changes may thus be a record of secretory activity in progress

In view of, firstly, the large body of evidence accumulated by other workers in favour of a correlation between cyto plasmic structures similar to lamellae and the associated fine particulate material and the basophilic properties of cells (e g Bernard *et al*, 1952 Weiss, 1953, Porter, 1954) and secondly as Jacobson and Webb (1952) have demonstrated that basophilia in osteoblasts is due to ribonucleoprotein, the lamellar regions in these cells may be those in which active synthesis of the specific material, of, for instance, the collagen protein fibrils is elaborated

A function of the amino polysaccharide component of the granules may be in connection with the formation of inter cellular material It has been shown that a non sulphated, hyaluronic acid like substance is present in the cultured osteoblasts during early development but that at a later stage when intercellular material has formed some sulphation has occurred This suggests that the non sulphated amino polysaccharide associated with protein as it is formed in the

closely associated with these collagen fibrils This stage in the morphogenesis of the bone must represent the beginning of calcification, it is probable that the electron opaque substance described is identical with that reported by Robinson and Watson (1955) in the rib cortex of infant bone and designated by them as a "calcification front"

Where the opaque substance has not masked clear definition, small crystals about 100 Å across are found to be localized almost exclusively between the *d* and *ab* bands (Schmitt and Gross notation, 1948) of the collagen fibrils (Fig 10) The number of crystals deposited in each interband, in the section of the fibril that is in view, varies from 1 to about 4 so far as can be observed, however, there is no preferred orientation of the crystallites relative to the fibril axis Where a greater degree of ossification has occurred the collagen fibres have become embedded in an opaque material which contains some crystals of similar size, and some periodic structure can also be distinguished (Fig 8) This intercellular material becomes quite compact and a remarkably sharp boundary is apparent between the ossified bone and the adjacent cartilage matrix (Fig 11)

(*c*) *Electron diffraction by the periosteal bone of the fowl embryo* Preliminary experiments indicate that the technique of electron diffraction may be a help in following the process of development of mineralization in bone Stripping of the bony sheath from embryonic long bones and the direct mounting of fragments of this material on electron microscope grids with or without a supporting film, have given interesting results

Powder diffraction patterns were obtained from 6½–12 day old embryos (Figs 12 and 13), measurements made on the spacings of these diffraction rings indicate that the diffracting material is an apatite Although the measurements are consistent with patterns obtained from a British Museum specimen of hydroxyapatite from Cherokee, Georgia they are not sufficiently accurate to define the diffracting material from the fowl embryos unequivocally as hydroxyapatite [it is now

formed collagen fibrils in direct association with the cells, however, where new material is being laid down, such a possibility is unlikely These newly formed collagen fibrils may be identical, however, with the "matrix precursor" which Leblond and co workers (1955) have postulated (from autoradiographs) as existing between osteoblasts and developing bone matrix

Fibrogenesis has been found to occur in intimate association with the cells and in periosteal bone the intercellular material is built up by apposition of successive layers of fully formed collagen fibres Considerable amounts of the osseous organic matrix are formed before the process of mineralization actually occurs

Young osteoid material taken directly from the embryo has been shown, by electron diffraction, to contain apatite This may be correlated with the particle formations found to be associated with the collagen fibres as seen in thin sectioned tissue At this embryonic stage, however, there is no evidence of any preferred orientation with respect to the fibre axis as was found in adult material by Finean and Engstrom (1953) by the use of low angle X ray scattering techniques The precise localization of the particles evident in the interbands, between the *d* and *ab* bands, of the collagen fibrils does not support the findings of Robinson and Watson (1955) who have associated bone crystals with the bands of collagen fibres in both infant and adult material

It is reasonable to assume that the opaque substance demonstrated in the thin sectioned material contains apatite, for this substance is found in regions where ossification is known to be occurring Clear definition of crystal formation in these regions has not been obtained, however, this suggests that the apatite may be in an amorphous state and crystallizes at a later date

Acknowledgement

The authors wish to express their thanks to Dr R H Smith and Mr R E Burge for discussion Mr G R Wilkinson for the infrared spectroscopy Mr K L Hull for help in the preparation of the plates and Miss J Harvey for preparative work with the bulk tissue cultures

cell is sulphated when it leaves the cell Such a mechanism may play a part in the transformation of precursors into the characteristic intercellular material It has been shown for instance, that normal bone matrix is usually composed of a sulphated polysaccharide (Belanger, 1954)

As the precise cytochemical localization of alkaline phosphatase coincides with that of the amino polysaccharide containing granules it must also be taken into account that the amino polysaccharide may be partially associated with the activity of this enzyme, for alkaline phosphatases are always found to contain polysaccharide (Schmidt and Thannhauser, 1943) The presence of alkaline phosphatase in the cytoplasmic granules and an oxidizing enzyme in association with them suggests strongly that the granules are centres of metabolic activity and that they are not just storage organelles

It has been shown by electron microscopy that during elaboration the intercellular material contains characteristic collagen fibrils which vary in diameter when seen in transverse section This indicates that the individual collagen fibrils of growing bones increase in size, which agrees with the general view that the fibrils increase in diameter once they have been formed (e g Porter, 1951, Randall *et al*, 1952) It has also been shown that each fibril is invested by interfibrillar material, in order to account for the enlargement of the fibrils this substance must therefore contain either collagen or the necessary precursors (Fitton Jackson 1956) Whether this interfibrillar material may be considered to be identical with the ground substance of bone is open to question, however, for the term "ground substance" is usually applied to the material occupying the spaces between the collagen bundles as seen by the light microscope

The fine structureless fibrils observed to lie at random in the developing bone matrix may be a component of typical ground substance The existence of such fibrils in cartilage matrix was described by Martin (1953) Alternatively, they may be immature collagen fibrils, in view of the appearance of fully

formed collagen fibrils in direct association with the cells, however, where new material is being laid down, such a possibility is unlikely These newly formed collagen fibrils may be identical, however, with the "matrix precursor" which Leblond and co workers (1955) have postulated (from autoradiographs) as existing between osteoblasts and developing bone matrix

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DISCUSSION

Meyer In our laboratory the people with whom I collaborate have grown fibroblasts from a great variety of sources and as far as we can tell the pattern of mucopolysaccharides is extremely similar they produce hyaluronic acid and chondroitin sulphate c the most prominent one being hyaluronic acid but they all produce a sulphated poly saccharide—this was long bone and parietal bone from human embryos and was compared to tendon to subcutaneum to a great variety of other tissues in mass culture I wonder whether you have any idea of what might be the identification of your periodic acid granules stain

Randall As we have shown the osteoblasts contain periodic acid Schiff positive granules in the cytoplasm When similar cells are grown in bulk culture and when these cells are harvested prior to the develop ment of extracellular material $CaCl_2$ extracts from these cultures demon strate the presence of a non sulphated hyaluronic acid like substance It is reasonable to relate this finding with the properties of the Schiff positive granules in the osteoblasts

Follis Would you contrast these findings with what you might expect to find in cultures of fibroblasts?

Randall The cytochemical properties of fibroblasts are identical with those of osteoblasts as far as we have been able to tell with the exception that the alkaline phosphatase activity is greater in osteoblasts than in fibroblasts

Follis Are you justified in calling these osteoblasts? You say that they come from explanted bone may they not have included fibroblasts?

Randall Organized growth may take place in the more central portion of a hanging drop culture In the cultures used in this work the cells differentiate into a tissue similar to that from which they were derived i e the osteogenic mesoderm of early fowl embryos which *in vivo* forms membrane bone behaves similarly when grown in culture

Lisco I was impressed by the colour of these pictures and it occurred to me that this material might be osteoid rather than calcified it was not strictly comparable to bone or let us say to calcified tissue

Follis It has more the appearance of callus to me

Randall Where can we draw the line? I am very interested to hear this

Follis The definition of osteoid tissue is whether it has the potentiality of calcifying otherwise it is collagen

Lisco Did you by any chance apply a silver stain to these cultures?

Randall Yes when differentiation has occurred in the cultures a positive result is obtained with the silver staining technique

Bélanger All these names may be interpreted differently but if we want to understand one another we must try to use them for the same thing! I do not think that the cells which we have been seeing in these cultures can be called osteoblasts They are possibly fibroblasts and as such may contribute to the formation of the fibrils The preosteoblast and the osteoblast contribute to the formation of the ground substance

at a later date and then the osteocyte proceeds to a modification or maintenance of that substance

Work of this type can contribute greatly to the understanding of the processes that lead up to the formation of bone tissue but not to the mechanism of bone formation and mineralization

Lisco It seems to me that we are dealing with a difficult problem of interpretation of certain facts and observations which up to a point are quite clear Prof Randall you have been showing pictures of cultures of cells that you call osteoblasts and that are derived from cells that have a potentiality of forming bone, but you have carried these cells only to a point where they are producing an intercellular substance of some sort beginning with intracellular globules and this you can stain with PAS I think from then on we disagree You say this is mineralized and I say it is not

Randall There is no doubt that the cultures grown from explanted membrane bone form bone It is also clear that we are not presenting any facts that demonstrate how the process of mineralization occurs In this work we are concerned with the earlier stage namely the formation of the intercellular organic material of bone the cytochemical properties of the cells have led us to a little better understanding of this process but we still have much to find out before attempting to relate the cytochemical properties of the cells as we know them now with mineralization

Amprino I think it is quite a debatable point whether in tissue cultures one can get true ossification or not for instance it is doubtful whether ossification takes place in explants made according to the hanging drop technique because in this case the cells commonly called osteoblasts migrate from the explant and their outer morphology changes completely furthermore it is not certain whether they can always lay down a matrix which is able to calcify Also, in normal bone in the living animal one can find osteoblasts which bear the characteristic microscopic features but there again spindle like cells seem at times to be associated with deposition of bone matrix that is new formation of bone may take place where the presumably osteogenic cells look like fibrobasts In conclusion *in vitro* as well as in the animal we have not always a clear picture of the so called osteoblasts in bone forming regions

Neuberger When you get these granules how do you know from which type of cell they come?

Randall The cytoplasmic granules are a general feature of fibrogenic cells concerned with the production of collagenous material It is for this reason that we have suggested that they may have fibrogenic properties The granules are of course present in fibrogenic cells *in vivo* but we have found that their behaviour in the cell can be investigated more accurately when similar tissues are grown in culture

THE MUCOPOLYSACCHARIDES OF BONE

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OUR group has been studying the types and quantities of acid mucopolysaccharides which can be isolated from various sources of connective tissue It is generally assumed that these acid mucopolysaccharides as protein complexes are components of the so called ground substances While this appears to be true for the non sulphated polysaccharides, the sulphated polysaccharides might be considered more appro priately as components of structural elements As far as I know, there is not very much information on the chemistry of the mucopolysaccharides of bone I shall not discuss the histochemical data on bone, because I believe that no state ment can be made on the nature of the polysaccharides on the basis of available histochemical methods Rogers (1951) reported the presence in ox shaft bone of 0 3–0 4 per cent of a sulphated polysaccharide, composed of about equimolar concentrations of hexosamine, uronic acid and sulphate, presumably a chondroitin sulphate However, only about 10 per cent of this amount was isolated Eastoe and Eastoe (1954) reported the isolation of a mucopolysaccharide protein complex obtained by lime water extraction of air dried bone powder (0 24 per cent by weight) On hydrolysis, they demonstrated both chondrosamine, 7 67 per cent, and glucosamine, 1 23 per cent, in this complex, and 1 63 per cent sulphate S On paper chromatography, they reported the presence of galactose, mannose and xylose

Our own work on bone is unfortunately still unfinished We wanted to answer the following questions

(1) What is the nature and the quantity of mucopolysac charides in adult and growing bone and how does the

pattern differ from cartilage and other connective tissue?

(2) Is there any indication of a phosphorylated polysaccharide as suggested by DiStefano, Neuman and Rouser (1953) in growing bone?

(3) What mucopolysaccharides are synthesized by fibroblasts of bone in tissue culture?

The answer to the second question is the simplest We have searched in vain for a phosphorylated polysaccharide in growing calf bone

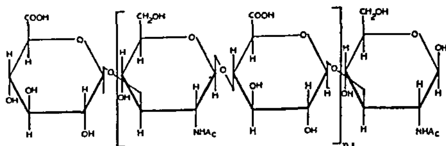


FIG 1 Structure of Hyaluronic Acid

The first two polysaccharides of Table I, hyaluronic acid and chondroitin, are sulphate free The two are isomers and their structures have now been established They contain repeating disaccharides, in the one case hyalobiuronic acid, in the other chondrosin of the structure of a β glucuronido 1 3 hexosamine They are polymerized without any demonstrable branching via β 1 4 linkages (Fig 1)

Next we come to the three chondroitin sulphates which we distinguish on the basis of their solubilities their optical rotation, their digestion by hyaluronidase and their colour reactions with carbazole and orcinole In our problem of bone and cartilage, chondroitin sulphate A and C are of interest B does not occur in cartilage or bone The structure of the repeating units of A and C are identical and known The

Table I

MUCOPOLYSACCHARIDES ISOLATED FROM CONNECTIVE TISSUES

Polysaccharide	*Gl am*	*Ch am*	*Uronic acid*	*Sulphate*	*Repeating unit*	*Hyaluronidase Action*		*Heparin activity*
						Testicular	*Bacterial*	
Hyaluronic acid	+		Glucuronic acid		HBA	+	+	
Chondroitin		+	Glucuronic acid		Chondrosin	+	+	
Chondroitin sulphate A		+	Glucuronic acid	+	Chondrosin	+		
Chondroitin sulphate C		+	Glucuronic acid	+	Chondrosin	+		
Chondroitin sulphate B		+	Unknown uronic acid	+	Unknown			+
Keratosulphate	+			+	Unknown			
Fractions related to heparin	+		Glucuronic acid (?)	±	Unknown			

HBA = Hyalobiuronic acid

sulphate group is presumably attached to carbon 6 of the chondrosamine and they are presumably polymerized through β 1 4 linkages (Fig 2) We do not know the cause of the difference between A and C

The next sulphated polysaccharide was named by us keratosulphate It is a polymer of a galactose, *N* acetyl glucosamine and sulphate Its structure is unknown We isolated it from cornea where it represents one half of the total mucopolysaccharides We now have isolated it or a fraction similar to keratosulphate from calf bone

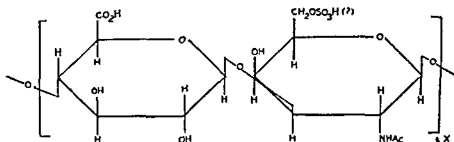


FIG 2 Repeating Unit of Cartilage Chondroitin Sulphate

The next fractions are highly interesting, though not in relation to bone These have been found by us in aorta They have in common a positive optical rotation, they contain glucosamine and a uronic acid which gives colour reactions different from the others and they are resistant to testicular hyaluronidase In all probability, they are related to heparin and they may originate from mast cells

Table II gives a summary of the distribution of the muco polysaccharides from different sources

Our data on bone are summarized in Tables III and IV As I have already mentioned, we hoped to find out whether growing bone contains polysaccharides which are absent in other connective tissues and absent in adult bone Let us take adult bone first From decalcified ox shaft bone powder (Table III) we isolated, as the only component, a polysac

Table II

DISTRIBUTION OF MUCOPOLYSACCHARIDES

Group I	Hyaluronic acid Vitreous humour synovial fluid tumours such as mesothelioma liposarcoma fowl leucosis Rous sarcoma Fujinami tumour
Group II	Chondroitin sulphate A and/or C Hyaline cartilage (trachea) chondrosarcoma, chordoma
Group III	Hyaluronic acid plus chondroitin sulphate C Primitive mesothelial tissue such as umbilical cord loose connective tissue of electric organ of eel
Group IV	Chondroitin sulphate B plus hyaluronate Skin
Group V	Chondroitin sulphate B plus C plus hyaluronate Tendon heart valves ligamentum nuchae
Group VI	Keratosulphate plus chondroitin sulphate A plus chondroitin Cornea
Group VII	Mucoitin sulphate of unknown structure Amyloid
Group VIII	Chondroitin sulphate A plus keratosulphate plus hyaluronate plus unidentified sulphated fractions Bone
Group IX	Hyaluronate plus chondroitin sulphate A or C plus B plus mucoitin sulphate of unknown structure Bovine aorta

charide in a yield of 250 mg per cent, which has the characteristics of chondroitin sulphate A in solubility, rotation, analysis, and enzymatic hydrolysis No fraction other than this one was evident

Table III

OX SHAFT BONE

TOTAL MUCOPOLYSACCHARIDE 250 MG PER CENT OF ACETONE DRIED DECALCIFIED POWDER

ANALYSIS (AFTER ADSORPTION ON DOWEX 1 × 1 COLUMN)

N	*Hexosamine*	*Uronic acid carbazole*	SO_4	$[\alpha]_D$
2.57	25.6 (0.78)	31.0 (0.87)	10.8 (0.61)	−31

Digested by testicular resistant to pneumococcal hyaluronidase

Table IV

FRACTIONS ISOLATED FROM CALF BONE

Fraction	*N*	*Hexos amine*	*Uronic acid*		SO_4	$[\alpha]_D$
			CO_2	*Dische*		
75 AI	2 67	26 1	33 6	20 0	13 5	−30
75 AII	2 85	28 1	33 8	33 3	10 5	−30
75 BI	2 81	26 3	—	32 0	10 1	−35
75 BIII	2 86	26 4	—	32 0	9 0	−29
83 AII*	2 88	30 4	33 6	31 9	12 0	−44†
83 AIII	2 87	25 8	32 5	31 0	13 3	−35
D V26 RII	3 66	24 3	—	1 5	12 9	−18**
{M VI 10A	3 27	39 1	—	43 2	<0 5	−70
{M VI 10B	2 39	26 4	—	29 0	11 6	−36

** Hexose (anthrone calculated as galactose) 25 6 per cent

† Paper chromatography chondrosamine 3+ glucosamine 2+ hydrolysed by both hyaluronidases

Our calf bone (Table IV) consisted of the distal ends of the long bones, frozen in the deep freeze, cut longitudinally and then by an electric jig saw trimmed to a thickness of 5–10 mm The material consisted of approximately 20 per cent epiphysis, the rest being cancellous and shaft bone From a histological point of view the starting material was quite a mixture The material was first ground in a chickenfeed mill and then in a power meat grinder and dehydrated with acetone and ether The dry weight was 35 per cent of the wet weight

We used two procedures In the first procedure, we brought the pH to 1 5 with HCl and digested with pepsin and trypsin to give Fractions 75 AI BIII (Table IV) A second batch was ground in a colloid mill to a fine powder with 0 1 N HCl and dialysed in the cold against 0 1 N HCl and then digested with pepsin and trypsin to give Fractions 83 AII and AIII There were no obvious gross differences between the products of the two procedures The yields are only approximate The total mucopolysaccharides isolated amounted to about 3 2 per cent The major part of the muco polysaccharide is chondroitin sulphate Fraction 83 AIII contained both glucosamine and chondrosamine and was

hydrolysed in part by pneumococcal hyaluronidase It was for these reasons suspected to contain hyaluronic acid mixed with sulphated compounds On fractionation with ammonium sulphate and pyridine, two fractions were obtained, one obviously hyaluronic acid M VI 10A, the other a chondroitin sulphate The total hyaluronate isolated from Fraction 83 AIII amounted to only ~280 mg We do not know how many of the other fractions contain hyaluronate

The high alcohol fractions not listed in Table IV gave a considerable anthrone reaction Electrophoretically, these high alcohol fractions contained a component with a slightly lower mobility than the chondroitin sulphate and similar to keratosulphate Attempts to separate this component on ion exchange resin were unsuccessful It was, therefore, subjected to hydrolysis with testicular enzyme to remove the chondroitin sulphate C which was obviously the main constituent On fractionation of the resulting digest, Fraction D V 26R was obtained which obviously is very similar to keratosulphate, that is it contains glucosamine, galactose and sulphate The yield was only ~ 250 mg

A major portion of the mucopolysaccharide fraction, (about one half of the total), which presumably is a mixture, has not yet been fractionated As far as we have determined thus far, this fraction contains no non sulphated components and presumably is a mixture of chondroitin sulphates which are not completely sulphated

Mass tissue cultures were grown by Drs Grossfeld and Godman The medium contained 10 per cent embryo extract in which about 4–5 mg of mucopolysaccharide is present, largely hyaluronic acid The feeding fluid, renewed every 4 5 days and pooled contained both hyaluronate and chondroitin sulphate in both cultures of long bone and of membranous bone There seemed to be little difference between the quantities of polysaccharides in long bone and membranous bone From 200 ml of the tissue growth (containing some fluid) 114 mg of polysaccharide were obtained from 740 ml of the culture fluid, 72 mg of polysaccharide were obtained,

i e , there was a far larger concentration of polysaccharide in the growth than in the culture fluid The higher concentration of polysaccharide in the cell growth may be significant, since with fibroblasts from other sources most of the polysaccharide was found in the feeding fluid

The polysaccharide fractions from cells and fluid were combined and the following fractions isolated

Table V

MUCOPOLYSACCHARIDES ISOLATED FROM TISSUE CULTURE (COMBINED CELL GROWTH AND CULTURE FLUID)

	Yield mg	*Hexos amine*	*Uronic acid*	SO_4	$[\alpha]_D$
I	26	31 5	37 3	2 4	−66
II	25	23 8	33 6	9 6	−25
III	7 2	—	30 1	—	—

Fraction I probably represents hyaluronic acid contaminated with some sulphated compound Fraction II probably represents chondroitin sulphate C contaminated with some hyaluronic acid Hyaluronic acid and chondroitin sulphate C have been isolated also from fibroblasts of subcutaneum and of tendon In these tissues, the hyaluronic acid fraction was many times that of the sulphated fractions In general, it appears that fibroblasts in tissue culture revert to the primitive type which produce preponderantly hyaluronic acid It should be pointed out here that Maurer and Hudack (1952) isolated hyaluronic acid from young callus in experimental fractures of rabbit femurs

In summary, the mucopolysaccharide pattern of growing bone is different from cartilage, in that in bone we have found hyaluronic acid and keratosulphate fractions which we have not found in hyaline cartilage Whether or not there are in bone, fractions typical for the tissue has not been determined

Acknowledgement

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DISCUSSION

de Bernard Do you think that β glucuronidase may be concerned with the synthesis of mucopolysaccharides?

Meyer No β glucuronidase has nothing to do with the synthesis of sugar, nor has it anything to do with the degradation of any of the polysaccharides

de Bernard Are these mucopolysaccharides protein free with regard to electrophoretic mobility?

Meyer Yes they do not give a Biuret reaction on analysis they appear to be protein free but of course this is within the limit of error of the method I would not say that all these preparations were necessarily even analytically protein free In cartilage and in bone the binding of chondroitin sulphate to protein is certainly of a labile type, but they are not bound by covalent bonds

Bélanger The keratosulphate that you are talking about is of course not related to keratin?

Meyer No We named this substance because it was first separated from cornea The name does not in any way imply that it is derived from keratin In the cornea keratosulphate is the main polysaccharide

de Bernard Are the different optical properties which you find in chondroitin sulphate *a* and *c* due to the degree of polymerization or to the molecular configuration?

Meyer I do not yet know We are investigating the structure of the chondroitin sulphates now

Neuberger Have you evidence of branching?

Meyer Yes at the present time this can be inferred only from enzymatic studies no other method is useful

Neuberger There is also a possibility that the sulphate group might be attached at different points either 4 or 6

Meyer Yes but the hydrolysis curve for the sulphate is the same in both for instance if one is a primary sulphate the other would be a secondary but no matter which one it is you would get differences in the hydrolysis

Neuberger How far according to the results at present obtained is the particular mixture of mucopolysaccharides and sulphates characteristic of a particular type of connective tissue? In fact if you were given a mixture could you guess from which type of connective tissue it is derived?

Meyer In skin and in cornea yes in bone we believe yes Of course this is mixed tissue it might be quite different if we took only shaft

bone or only the plates in the epiphysis In tendon maybe In heart valve I believe yes We have investigated only one species of heart valve i e pig heart valve In skin chondroitin sulphate is the main polysaccharide but there is much more chondroitin sulphate seen in rodent skin than there is in pig skin Pig skin yes We have not investi gated human skin This is the work of a group with which I am con nected

Kodicel I would like to put to you in a slightly different form the question which I asked Prof Lacroix what makes the bone calcify in comparison to the tendon? From your results can you say that the difference in mucopolysaccharide set up would justify our saying that the mucopolysaccharides are responsible for calcification?

Meyer I cannot say this There is a large fraction and we do not know what it is This fraction is characterized by the fact that it is remarkably undersulphated it does not contain chondroitin at least as far as we can tell from the ion exchange experiments which distinguish very beautifully between sulphated and non sulphated polysaccharides I presume that this is the most interesting fraction we suspect it is the fraction which picks up the sulphate which shows up as radioactive sulphate in various areas as demonstrated by a number of people here This could only be demonstrated in a cell free isolated enzyme system and I wish someone would do this

AUTORADIOGRAPHIC STUDIES OF THE FORMATION OF THE ORGANIC MATRIX OF CARTILAGE, BONE AND THE TISSUES OF TEETH

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Introduction

SOME 150 years have already gone by since John Hunter (1778) informed us that when the "bony part" of the teeth was burned, it blackened and when its salts were removed by acid, there remained a "gristly or fleshy" residue Much has been learned in the meantime about this gristly, fleshy, blackening substance In bone and also in dentine and cementum, the fibrillar albuminoid collagen has been identified and even visualized (Ruth, 1947, Robinson and Watson, 1952) as forming a skeleton to the skeleton, becoming ensheathed by the crystals and an amorphous organic filler, the ground substance (Robinson, 1952, McLean and Urist, 1955) The solid portion of ground substance has been found to contain mainly carbohydrates in the form of polysaccharides (Wislocki, Singer and Waldo, 1948, Wislocki and Sognnaes, 1950, Bevelander and Johnson, 1955), generally complexed with proteins An acid fraction contains glucuronic acid (Glegg, Eidinger and Leblond, 1954) and has been identified as chondroitin sulphate and produces metachromasia with the thiazine dyes (Follis, 1949, Bevelander and Johnson, 1955, Levine and Schubert, 1952) Another fraction termed neutral (Meyer, 1945) contains adjacent hydroxyl or hydroxyl-amino groups in the sugar moiety which are oxidized to aldehyde and produce a colour reaction when the periodic acid Schiff (PAS) test of Hotchkiss (1948) is applied (Glegg,

Eidinger and Leblond, 1954, Leblond, 1950) Evidence of an association between the neutral polysaccharide fraction and alkaline phosphatase has been found (Moog and Wenger, 1952)

Cartilage contains the same organic constituents with a characteristically large proportion of various sulphated muco polysaccharides (Meyer and Rapport, 1951)

The skeleton of enamel has been described as an ectodermic albuminoid, keratin (Leicester, 1949) This keratin has a low sulphur content (Leicester, 1949) and according to Pincus (1936) and Block (1951) shows only traces of cystine upon hydrolysis Pincus (1949) has also reported the presence of a mucoprotein, which might be some sort of a ground substance constituent, rendering enamel comparable to the other miner alizing tissues at least in its early form

Quantitative estimates in stained preparations by Sylven (1948) have revealed a decrease of the sulphated muco polysaccharides in cartilage at the time of mineralization A decrease in stainability of the maturing bone matrix by the Hotchkiss process after periodic acid has also been reported by Heller Steinberg (1951) and related to progressive poly merization of the neutral polysaccharides, according to the theory of Gersh and Catchpole (1949) The same author (Heller Steinberg, 1951) has observed an intensified colour reaction in areas of bone growth and destruction, attributed in both cases to the presence of low polymers A cell matrix sequence based on the detection of PAS positive material in the osteoblasts (Heller Steinberg, 1951) and the odonto blasts (Bevelander and Johnson, 1955) has been reported in the elaboration of the neutral polysaccharides but not for the acid mucopolysaccharides (Bevelander and Johnson 1955)

The dynamics of secretion and metabolism are now being gradually uncovered by the use of radioactive tracers Although non specific the ^{14}C mapping by Greulich and Leblond (1953, 1954) has revealed a sequence in the production of organic matrix in cartilage bone and dentine With the help of $^{35}SO_4$ Dziewiatkowski (1951*b* 1952) and also Bostrom

and Odeblad (1953) have revealed the entry of a sulphated compound in cartilage which these authors (Dziewiatkowski, 1951*a*, Boström, 1953) have separately isolated as chondroitin sulphate Dziewiatkowski (1952) has also observed that the rate of chondroitin sulphate turnover was highest "in the epiphyseal region adjacent to the diaphysis and to the secondary centers of ossification", inferring that calcification is associated with an accelerated synthesis and removal of chondroitin sulphate In the diaphyseal bone (Dziewiatkowski, 1952), he has observed a progressive increase of ^{35}S towards the mid diaphysis which seemed to disappear more rapidly than the same material at the "ends of the diaphysis"

Materials and Techniques

The autoradiographic observations on cartilage bone, dentine and enamel of young rats and hamsters (Belanger, 1954*a*, 1955*a*) reported here were initiated in 1953 ^{35}S was injected at first as $H_2{}^{35}SO_4$, 5 μc/g weight The tissues were fixed in neutral formaldehyde ethanol and embedded in low viscosity nitrocellulose "Integrated autoradiographs" were obtained by coating with fluid emulsion from High Contrast Positive Film (Eastman Kodak Co) and staining the subsequently inverted specimens with 0 02 per cent aqueous basic fuchsin (Belanger, 1950, 1952*b*, Belanger and Leblond, 1946) These experiments were undertaken in order to study the formation and evolution of the sulphated fraction of the polysaccharides

Comparable series of autoradiographic mapping were undertaken at a later date, with the help of biosynthesized ^{35}S cystine and methionine (Belanger, 1955*b*) Since hydrolysates of collagen reveal practically no cystine and very little methionine (Hawk, Oser and Summerson, 1954), the images obtained should be related mainly to the glycoprotein fraction in cartilage, bone and dentine Enamel, of course, was expected to reveal preferentially new keratin synthesized with the ^{35}S amino acids

Results

Cartilage and Bone

The heads of the long bones have revealed a definite cellular localization in the early records of the radiosulphate series (Fig 1) There was a gradient of intensity of the auto radiograph increasing from the articular surface towards the hypertrophic cells Below this area, the image was practic ally negative over the zone of calcification where very few intact cartilage cells were visible The bone spicules at the epiphyseal plate showed a weak but definite image In the newly formed spicules and also at the diaphyseal area of growth (Fig 6), a gradient in the autoradiographic record was apparent, showing an increasing concentration of radioactive material with maturation The presence of radiosulphate in concentrations visually less than those of cartilage and bone was revealed in tendons, ligaments and muscular aponeuroses

At 1 day and also at 2 days (Fig 2) radioactive sulphur was still detected over the cartilage cells but an increasing pro portion was recognized over the intercellular substance Some of this radioactive matrix had now reached the zone of calcification by the continuous process of growth

Over the bone, a general decrease in the intensity of the autoradiographic image had then taken place as if most of the material recorded at 2 hours had been labile Definite bands were visible along the areas of apposition at some distance from the surface in a pattern comparable to that previously described for mineral deposition (Leblond *et al*, 1950) These tagged portions of tissue have probably been deposited at the time and at the place of new bone formation and are now being displaced by the continuous process of growth In the diaphysis (Fig 8), the band exhibits an arciform pattern with the apex at the mid diaphysis

At 6 (Figs 3 and 4) and 8 days after the introduction of tracer, the cartilaginous head of the long bones exhibited a strictly extracellular localization of the radiosulphate The new bone spicules growing at the epiphyseal plate (Figs 3 and 4) were apparently more radioactive than those of the

animals sacrificed early (Figs 1 and 2) At high magnification (Fig 4), the radioactivity seemed to be distributed throughout the spicule

A few sections of the above described tissues were placed in a demineralizing bath of formic citrate at pH 4 9 for 24 hours They were then negative to the silver nitrate test Autoradiographs of such demineralized sections revealed practically no change in the cartilage picture On the contrary, the original diffuse distribution of $^{35}SO_4$ over the bone had disappeared and thus seemed related to some intake or exchange in the mineral fraction A weak band image (Figs 7 and 8) persisted in the areas of growth (metaphyseal and diaphyseal), it was interpreted as representing some newly synthesized component of the matrix, most likely chondroitin sulphate

Other sections were incubated in a 37° bath of 0 1 per cent hyaluronidase at pH 5 8 for periods of 1 to 6 hours This treatment produced a progressive loss of radioactive material from the cartilage head except in the areas of tendon and ligament insertions (Fig 5) These areas which were previously invisible after toluidine blue, stained metachromatically after hyaluronidase treatment These localizations were interpreted as regions of high mucopolysaccharide concentration or otherwise, possible concentrations of hyaluronidase-resistant chondroitin sulphate, type B (Meyer and Rapport, 1951)

The observations made with ^{35}S methionine on cartilage and bone have revealed some comparable events and also some altogether different occurrences This amino acid has produced at the level of the hypertrophic cells of the cartilage head, a cellular synthesis record progressively increasing in intensity from 1 hour (Fig 9) to 6 hours (Fig 10) to 2 days (Fig 11) Other portions of the cartilaginous tissue did not seem to exhibit radioactivity, except the region immediately surrounding the epiphyseal centre of ossification at 6 hours (Fig 10) which is then homologous with the zone of hypertrophic cartilage

The most intense region of the head of the bone was not the above mentioned but the well delimited zone of penetration leading to the formation of the epiphyseal centre of ossification, at all stages described above (Figs 9, 10, 11) Also very active were the formative zones of the bone (epiphyseal plate, metaphysis, diaphysis) At high power the reaction appeared to involve the osteoblasts Since the invasive zones of the head would contain especially cells which could be termed preosteoblasts it seems that a definite cellular stage has been demonstrated in the formation of bone matrix substance from radiomethionine and cystine This cellular phase was never visualized in the radiosulphate series

Other primary localizations of interest in the methionine experiments were the areas known as "l'encoche de Ranvier" (Lacroix, 1949) where intense cartilage formation occurs (Figs 9, 10, 11), also the haemopoietic marrow and the lesser radioactive envelope of the cartilage head, the tendons, ligaments and the striated muscle, most intense were the keratogenous zones of skin and hair (Belanger, 1955*b*)

The animals injected with radiocystine have shown a comparable pattern of initial distribution with a slower entry related to the low solubility of this amino acid

The sections were generally most radioactive at 2 days Extracellular radioactive material was apparent at this stage in the cartilage matrix of the hypertrophic zone (Fig 11) The newly formed spicules at the epiphyseal plate were relatively much less active than the distal portion formed at the time of injection (arrow, Fig 11)

At 4 days (Fig 12), the cartilage head showed relatively little activity, all of it extracellular and diffusely distributed The epiphyseal plate and the bone marrow were practically negative, the tagged elements having now disappeared except from laterally incorporated spicules (arrow, Fig 12) The diaphysis (Fig 13) revealed an arciform band of incorporated material which had lost little of its original activity and had been mobilized in a manner comparable to that of the radiosulphate tagged band







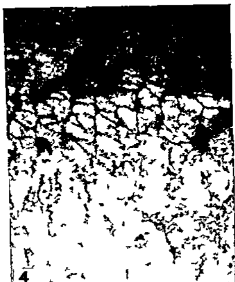
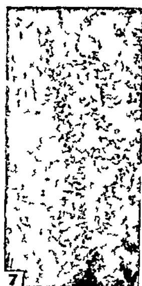


PLATE I ^{35}S Sulphate

Fig 1 Head of humerus rat 2 hours × 32
Fig 2 Lower extremity of femur rat 2 days × 32
Fig 3 Upper extremity of femur rat 6 days × 32
Fig 4 Epiphyseal plate upper extremity of tibia rat 6 days × 180







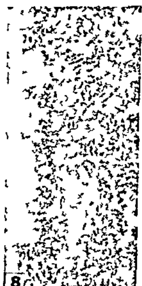


PLATE II ^{35}S Sulphate

FIG 5 Lower extremity of humerus rat 24 hours Hyaluronidase 3 hours × 32

FIG 6 Diaphysis of humerus rat 2 hours mineralized section × 110

FIG 7 Funnel area rat 2 hours demineralized section × 180

FIG 8 Diaphysis rat 2 hours demineralized section × 110

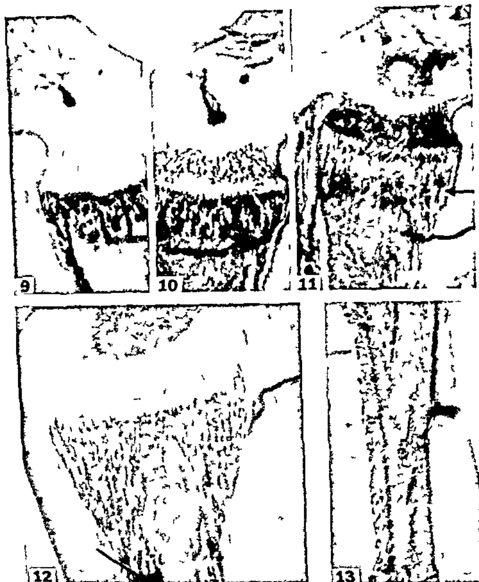


PLATE III ^{35}S Methionine

Fig 9 Head of tibia rat 1 hour × 18
Fig 10 Head of tibia rat 6 hours × 18
Fig 11 Head of tibia rat 2 days arrow = down growth of spicules × 18
Fig 12 Head of tibia rat 4 days arrow = incorporated spicules × 18
Fig 13 Diaphysis of tibia rat 4 days × 18









PLATE IV ^{35}S Sulphate

FIG 14 Incisor rat 2 hours mineralized section × 110 G = gap
FIG 15 Molar rat 2 hours demineralized section × 360 E = enamel PD = predentine
FIG 16 Incisor pig 4 minutes i v × 100 D C = dentine cementum junction
FIG 17 Incisor pig fluorine 1000 p p m 31 days 8 minutes i v × 100









PLATE V ^{35}S Sulphate Cystine

FIG 18 Molar rat ^{35}S sulphate 4 days × 110
FIG 19 Molar rat ^{35}S sulphate 6 days × 110
FIG 20 Molar rat ^{35}S sulphate 24 hours hyaluronidase 3 hours × 360
FIG 21 Molar rat ^{35}S cystine 24 hours × 15









PLATE VI ^{35}S Methionine

FIG 22 Second and first molars rat 2 hours × 32 G = gap
FIG 23 Second molar rat 2 days × 3[illegible]
FIG 24 Molar rat 4 days short exposure × 60
FIG 25 Molar rat 4 days long exposure × 60

Dentine

The 2 hour autoradiographs of mineralized dentine (Fig 14) in the radiosulphate survey with young rats, were quite comparable to those of diaphyseal bone (Fig 6) They showed a diffuse, general intake of ^{35}S which seemed to increase to a maximum and then decrease over the more mature tissue A comparable pattern had previously been recorded in ^{32}P exchange experiments *in vitro* (Bélanger, 1953) favouring a similar interpretation in this case The tissue of the pulp chamber produced a diffuse autoradiographic record of less intensity than that of dentine Certain areas, such as the uppermost portion of the chamber in the crown and a wide irregular transverse band at the entry of the pulp chamber displayed larger concentrations of radioactive material No record related specifically to the odontoblasts was apparent at any time

After acid demineralization of the slide (Fig 15), the general reaction over dentine was replaced by a band definitely located to predentine and apparently of even intensity throughout

The initial maximum concentration of radiosulphate in predentine was confirmed in a recent study carried out with pigs fed with various amounts of fluorine and sacrificed at graded intervals after administration of a single dose of radiosulphate This work was a pendant to a previous under taking by Dr C L Comar and his associates at the University of Tennessee A E C Agricultural Experiment Station (1952, 1953, Bélanger *et al*, 1954) in which the deposition of minerals was studied with ^{45}Ca as tracer In that survey and also in the present one, the teeth were processed and observed in our laboratory The incisors of animals sacrificed 4–8 minutes after an intravenous dose of radiosulphate have revealed that most of the radioactive material entering through the pulp was located in predentine (Figs 16, 17) The junction of the cementum and dentine produced also an autoradiographic record but of lesser intensity (Fig 16) Observations on the rat had revealed an image at the dentine enamel junction in

the older animals whose teeth were studied after grinding (Bélanger, 1955*a*)

The fluorinated teeth with their characteristically enlarged, irregular and wavy predentine (Fig 17) seemed to take up a larger amount of radiosulphate than the normal teeth (Fig 16)

At later intervals (Figs 18 and 19), the radioactive band was displaced upwards by further apposition of non radio active matrix and with a progressive, slow loss of intensity The diffuse distribution of the radiosulphate was also visual ized *in the dentine and alveolar bone of the pig fluorine* specimens The pulp picture persisted intact after acid treatment

In the cystine methionine series, the early autoradiographs showed that the radioactive material was originally taken up by the formative cells (odontoblasts and preodontoblasts of the root, Fig 21) At later intervals (Fig 25) a definite incremental band was recorded over the dentine at a progres sively increasing distance from the pulp border

Enamel

An unexpected finding during the radiosulphate mapping of the teeth, was a strong record at the level of enamel At 2 hours after ^{35}S administration, the radiosulphate ap peared as a thin band located at the pre enamel (Figs 14, 15, Orban, 1949) but only in the regions facing the large, secretory ameloblasts Sometimes, a short portion of detached enamel organ ("gap") marked the end of the secretory phase (G, Fig 14, Marsland, 1951 Belanger, 1955*a*) A reaction of lower intensity could also be picked up at this stage over the secretory large ameloblasts

The reaction of pre enamel was not affected by acid demineralization (Fig 15) Hyaluronidase incubation of the sections, as described above, did not affect the radiosulphate content of enamel in treatments lasting up to 6 hours (Fig 20) although in such slides no record was obtained from the adjacent cartilage, bone or dentine Furthermore, in the

same sections of the heads of young rats and hamsters, a strong record persisted over the mucous cells of the salivary glands present (Bélanger, 1954b)

At 2 and 4 days, the radiosulphate tagged substance was displaced away from the ameloblasts by the addition of more pre enamel and appeared within the growing enamel at a rate inversely proportional to the actual age of the tissue (Fig 18) Meanwhile, the definite band first observed in pre enamel became progressively more diffuse and less intense At 6 days (Fig 19) the enamel record, previously much stronger, had become weaker than that of dentine This observation points to a rapid metabolism of the enamel sulphate compound

The cystine methionine series also revealed some rather surprising facts in regard to enamel While the teeth of animals treated with ^{35}S methionine produced intense auto radiographs over the enamel (Figs 22, 23, 24, 25), those treated with ^{35}S cystine (Fig 21) revealed practically no intake by this tissue In contrast, the skin and other histo logical entities (Fig 21) demonstrated a comparable intake and subsequent behaviour (Belanger, 1955b) These findings seem to confirm previous reports of a low cystine content in enamel (Pincus, 1936, Block, 1951)

The early autoradiographs of enamel after ^{35}S methionine have revealed considerable differences in intake by young and older portions of the tissue the whole enamel of the young tooth (Fig 22) is totally and diffusedly invaded by the radio active substance within the first 2 hours following the subcutaneous introduction of tracer while the older tissue (Fig 22) is invested in a superficial fashion only (pre enamel) with the tracer material The secretory relationship with the large ameloblasts recognized in the radiosulphate approach, has also been evidenced in the methionine material A sudden cessation of intake has been visualized at the level of the enamel "gap" (G, Fig 22)

At 2 and 4 days (Figs 23 24) inward displacement of the originally tagged zone has been recorded as well as differences in the regional content of radioactive substance At the same

time, when the position of the "gap" (G_1, Fig 23) was observed in relation to the original limit of radioactive intake, a retrograde movement of this landmark (from G to G_1, Fig 23) could be visualized This phenomenon points to a progressive change *in the ameloblasts and is part of the physiological* behaviour of the enamel organ leading to the formation of an optimal layer of tissue

In autoradiographs of long exposure (Fig 25), all of the enamel deposited up to 4 days after the introduction of tracer, has shown the presence of radioactive material as if there had been continuous entry or local redistribution, but practically no radioactive material seemed to have penetrated the preformed enamel

Autoradiographs of 2 and 4 day animals (Figs 23, 24, 25) have shown practically no loss of radioactive material over *enamel, an indication of a slow metabolic turnover of the* substance thus tagged

Summary and Conclusions

Autoradiographic studies with ^{35}S sulphate have revealed a rapid synthesis of a hyaluronidase labile fraction in the matrix of cartilage, new bone and new dentine and of a hyaluronidase resistant substance in new enamel A minor hyaluronidase resistant fraction related to the insertions of tendons and ligaments was also demonstrated in cartilage

A secretory phase has been visualized in cartilage and in the enamel organ (large chondrocytes and large ameloblasts)

In all tissues radiosulphate was initially most abundant in *the non mineralized areas (hypertrophic cartilage pre bone,* predentine, pre enamel)

With time, there was a progressive diffusion into the matrices *and subsequent disappearance of the sulphate tagged* substances most rapid in the enamel

^{35}S methionine has also been traced into cartilage bone, dentine and enamel *Autoradiographs* prepared soon after treatment, have revealed the presence of radioactivity over

the formative cells, even the very young (prechondroblasts, preosteoblasts)

The early concentration of the methionine tagged material has been found proportional to the growth rate in bone and enamel

In bone, dentine and enamel, the radioactive material has been deposited as a layer at the accretion zones At later intervals it retained a band like appearance in the auto radiograph and became displaced inwards by the continued growth process With the exception of cartilage, very little loss of radioactivity with time could be visualized in the autoradiographs of mineralizing tissues

Methionine and cystine labelled animals have given a similar pattern of entry and transit as visualized by autoradiography with the exception of enamel from which no record has been obtained after administration of radiocystine

In the present series of observations, it seems that the methionine labelled fraction is different from the sulphate labelled fraction The former probably predominates in bone, dentine and enamel, by accumulation in the mineralized portion of the tissue

In enamel, the methionine tagged fraction presumably represents newly synthesized keratin In view of the low sulphur content of collagen, it is likely that most of the methionine in cartilage, bone and dentine has been incorporated into the glycoprotein complex containing the neutral polysaccharides The formative cells which gave an auto radiographic record with ^{35}S methionine and cystine, have already been reported to give a positive PAS

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Fig 1 2 3 5 6 18 and 19 are reproduced with authorization from the Canadian Journal of Physiology and Biochemistry Figs 9 10 11 13 22 23 24 and 25 with authorization from the Anatomical Record

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DISCUSSION

Randall What evidence have you that methionine stays as methionine and does not go into some pool or other before it reaches the radioactive part of the tissue which we have described?

Bélanger I have no evidence whatever When ^{35}S is introduced as an amino acid there are not many things that can happen to it, a little can be oxidized to sulphate most of it is built up into polypeptides and proteins so the largest fraction retained after histological treatment is likely to be the protein fraction Whether it has been tagged with methionine itself I do not know In a very recent experiment done just before I left Canada we exposed sections of bone thus labelled to hyaluronidase There was no loss of radioactivity

Neuberger I think the interpretation of these experiments would depend to a large extent on the dose of methionine given Perhaps I should amplify that statement supposing you had a diet just supplying enough methionine for building up new proteins you would probably get relatively very little formed into sulphate On the other hand if you were to give a large dose of methionine which the body could not utilize most of the methionine S would be transformed into sulphate and you should therefore get the superimposition of the sulphate picture on your methionine picture

The other point is that if you give methionine a certain amount of the S will be converted to cysteine independently of the dose In fact I do not think that you can give a dose of methionine without having a considerable proportion transformed into cysteine *vice versa* of course if you give cysteine you know that none or very little of it will go into methionine You would expect therefore a difference between the methionine and cysteine pictures The question of dose seems to me quite important What was the general protein content of the diet and how much extra methionine was supplied?

Bélanger The animals under treatment were suckling rats which were dosed at four days of age and sacrificed at intervals up to the fifteenth day so all the protein they were getting was from mother rat When compared to litter mates they showed no difference in the rate of growth ^{35}S methionine was given in a dosage of 3 μc/g weight

Dent I wonder what chemists think about taurine as a possible metabolite because taurine crops up in extraordinary places

Neuberger What about glutathione? You are bound to have a certain amount of glutathione there It turns over very much faster than a protein You may have a very high rate of activity in your glutathione Is that a possibility?

Bélanger That is for the chemists to interpret

Neuberger I think you cannot make the assumption that if you give methionine or cysteine and you find it in certain tissues it would neces sarily be in the form of tissue protein There are certainly plasma pro teins in connective tissue and there are various other soluble substances which have a fairly fast turnover I do not think that collagen is the only possibility

Blaxter Is it possible to demonstrate sulphydryl groups histo chemically and if so do they show a correlation with the distribution of the radioactive methionine?

Bélanger There are various histochemical techniques for sulphy dryls it is up to the chemists to let me know whether they are good or not There are variations in the literature in regard to the localization of sulphydryls depending on what technique has been utilized In the present case no histochemical investigation of the sulphydryl groups has been carried out

Meyer The fact that your tissue has gone through a fixation would mean that the very soluble glutathione and methionine unless the methionine were bound to protein would be removed and since this involves a process of fixation only the fixed non soluble protein plus whatever else is insoluble or has become insoluble would be left over So I think that most of the radioactivity which was shown regardless of what it is chemically is certainly not a low molecular water soluble fraction

Kodicek Wood from Memphis has done some work on radiomethio nine in granulation tissue and he also came to the conclusion that there is a very low incorporation I think only about one per cent of the dose went into the granulation tissue and it was mainly in the non soluble protein fraction

Armstrong Is there displacement of the cartilage cells?

Bélanger There is certainly a growth movement of the cartilage towards the plate but if we obtain autoradiographs within a few hours I do not think that we are dealing with a displacement since it takes two days before we can see radioactivity within the calcified zone devoid of secretory activity Before we see evidence of radioactive material in that area I would assume that what we are visualizing is a passage from the cells towards the matrix The subsequent local decrease in radioactivity can be ascribed to an actual turnover or utilization of the tagged substance

UPTAKE OF ^{35}S IN THE DIFFERENTIATION AND GROWTH OF CARTILAGE AND BONE

RODOLFO AMPRINO

Institute of Human Anatomy University of Bari

RADIOSULPHUR* is a unique and almost ideal tool in the study of the formation and metabolism of ground substance in supporting tissues In fact, this radio isotope seems to be incorporated almost exclusively in the synthesis of sulpho mucopolysaccharides (*Dziewiatkowski, 1951, Boström and Åqvist, 1952, Boström and Mansson, 1952*) Furthermore, autoradiography provides the means for investigating the distribution and to some extent also the relative concentration of sulphomucopolysaccharides in the tissues in relation to the histological structure of the latter

The study of cartilage in connection with the metabolism of sulphomucopolysaccharides in supporting tissues is of special interest, not only because cartilage represents the richest store of chondroitin sulphate in the whole organism, but also because of the intimate relationship which seems to exist between cartilage and bone in some areas of the body In perichondral and endochondral ossification, bone envelops and gradually replaces cartilage, but so far the true nature of this relationship is more a matter for speculation than of actual knowledge Both in ontogenesis and phylogenesis a merely topographical relationship between the two might exist, or else a more intimate connection, e g , a biochemical dependence of bone formation on cartilage Lacroix (1949) maintains, for instance that a substance freed from cartilage,

* Radiosulphur is incorporated into the ground substance of the mesenchymal derivates when administered either as inorganic sodium ^{35}S sulphate or ^{35}S labelled cystine or methionine (Bélanger 1955)

Dent I wonder what chemists think about taurine as a possible metabolite because taurine crops up in extraordinary places

Neuberger What about glutathione? You are bound to have a certain amount of glutathione there It turns over very much faster than a protein You may have a very high rate of activity in your glutathione Is that a possibility?

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Neuberger I think you cannot make the assumption that if you give methionine or cysteine and you find it in certain tissues it would necessarily be in the form of tissue protein There are certainly plasma proteins in connective tissue and there are various other soluble substances which have a fairly fast turnover I do not think that collagen is the only possibility

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certain that the high uptake of sulphur is mostly due to its incorporation in the cells, from which the matrix is subsequently elaborated, the latter soon becomes basophilic and metachromatic From this stage onward, the amount, the basophilia and the metachromasia of the matrix increase rapidly and steadily, radiosulphur uptake runs parallel with the increment of intercellular substance and its increase in basophilia

In an autoradiograph the precartilaginous blastema is not sharply differentiated from the surrounding less radioactive tissue, the transition between the two is indicated by a narrow fringe which corresponds to the layer of mesenchymal cells which are gradually undergoing determination and transformation into precartilaginous elements Still later, this less radioactive rim is represented by the perichondral envelope, in tangential sections, autoradiography shows that the sulphate uptake in the perichondrium is much lower than in the cartilage proper but distinctly higher than in the adjacent connective tissue which will not be assimilated by the cartilage during its appositional growth

The rate of radiosulphur incorporation into the cartilage increases with the progress of differentiation and inner growth processes In mammalian foetuses, the uptake of radiosulphur increases appreciably in the regions in which chondrocytes undergo hypertrophy, this behaviour is readily seen in the diaphyseal aspect of epiphyseal cartilages In the zone of proliferating cells, uptake is relatively lower than in the adjacent layer where chondrocytes are separated from one another by large amounts of intercellular basophilic matrix In agreement with Dziewiatkowski's findings (1951) radioactivity is much higher in the region of swollen chondrocytes which still multiply and in the hypertrophic layer proper than in the zone in which disintegration of the intercellular septa begins Notwithstanding the relative reduction in thickness of the septa radiosulphur uptake is very high in the hypertrophic zone of the epiphyseal plate, probably a rapid synthesis with renewal of chondroitin sulphate occurs there

"osteogenin", may evoke the osteogenetic activity of connective cells Other authors are of the opinion that some constituents of the cartilage matrix could be utilized in the synthesis of organic bone matrix (Siffert, 1951, Godard, 1951)

The participation of the cellular components in the production of intercellular matter has long been postulated It has been shown that the formation of metaplastic substances ceases on the death of the cells However, direct proof that cells play a basic rôle in the first steps of matrix production was recently provided by Belanger (1954), by means of autoradiographic investigations after administration of ^{35}S sulphate Pelc and Glucksmann (1955) have independently given an analogous demonstration in the hyaline cartilage in young adult mice, the latter authors found that in the xiphoid process in which the chondrocyte is almost completely filled by a large fat drop, the autoradiograph made two hours after ^{35}S administration is most intense over the thin peripheral rim of cytoplasm and it appears as a ring twenty four hours after treatment, the radioactivity diminishes over the cell and increases over the matrix

Apparently, the same pattern in the formation of ground substance can be traced in the first steps of cartilage histogenesis during embryonic development In this connection, it is of some interest that of all the mesenchyme which will give rise to the various supporting tissues, to smooth muscle cells, etc, the part which is destined for differentiation into cartilage can be identified by its capacity for sulphur incorporation before the onset of any microscopical differentiation Already in 5 to 6 day old chick embryos, the precartilaginous blastema composed of irregularly shaped and closely packed cells, among which an acidophilous, not yet metachromatic, tenuous network of interwoven collagen fibres is apparent shows a relatively higher radioactivity after ^{35}S treatment than do neighbouring mesenchymal areas from which subcutaneous tissue or derm will form In the precartilaginous blastema, the cells represent by far the most marked component while the intercellular matrix is still scarce It seems

cartilage is invaded by the fibrovascular buds and resorbed Short term experiments (24 hours), show a conspicuous radio activity even in the fringe of cartilage which is being resorbed and replaced by bone tissue On the other hand, the sulpho mucopolysaccharide formed before the onset of ossification is apparently freed from the matrix before the breakdown of the latter, in fact, if treatment with radiosulphur is started several days prior to the beginning of endochondral bone formation, no radioactivity is detectable in those regions which appear intensely radioactive after a short treatment In other words, a renewal of chondroitin sulphate seems to occur in the matrix of vertebrae but not in the cartilaginous diaphysis of long bones in chick embryos The essential difference between the cartilage of short bones and that of the diaphysis of long bones in the chick is that the former calcifies before being resorbed while the latter does not Furthermore, the cartilage of the diaphysis is not replaced by endochondral bone trabeculae, instead, the cartilage of the vertebral body and processes undergoes substitution by bone tissue, at least to some extent

Some time ago, Logan (1935) stressed the fact that calcification of cartilage is preceded by a loss of organic sulphate This relationship was later emphasized by Hass (1943), who suggested that the maintenance of a high level of chondroitin sulphate is a device by which cartilage is protected against calcification Dziewiatkowski (1951, 1954), Davies and Young (1954), and Belanger (1954) conclude from their autoradiographical studies after ^{35}S administration that a connection seems to exist between the laying down of bone tissue and depletion of sulphate of the cartilage matrix in regions of endochondral ossification in mammals the sulphur containing components laid down in the areas of calcification may get their sulphur from the chondroitin sulphate of the adjacent cartilage as well as from circulating inorganic sulphate S (Dziewiatkowski, 1952)

The above mentioned results on the differences in the chondroitin sulphate metabolism in the cartilaginous anlage of long and of short bones in the chick seem to represent

Great variations in the rate of synthesis of chondroitin sulphate within any given cartilage occur also in the anlage of long bones in bird embryos (Amprino, 1955) This was shown by studying chick embryos at various intervals after ^{35}S administration e g, *from 2 hours to 10 days (short and long term experiments respectively)* In some regions of the cartilaginous anlage, synthesis may cease, and conversely be very marked in neighbouring regions, for instance, it decreases rather suddenly in the diaphysis when the peri chondral bone ring is laid down, while it increases progres sively and remains intense for a long time in the metaphysis In the epiphysis proper, radiosulphur uptake is, in general, lower than in the adjacent metaphysis, this difference becomes more marked in late developmental stages, e g, from the fourteenth day of incubation to hatching time (twenty first day) In this regard, it must be stressed that ossification of the epiphyseal cartilages in the chick takes place only in a rela tively late period of postnatal growth

The decrease of sulphur uptake in the cartilaginous dia physis of the anlage of long bones and its final cessation when the fibrovascular buds penetrate into the cartilage through the perichondral bone ring, is a constant finding in the chick No evidence has been found, however, of any depletion of the chondroitin sulphate previously synthesized in this region In fact, long term experiments show that the radioactivity of the cartilaginous diaphysis—which depends on the radio sulphate incorporated during several days—does not diminish when the perichondral bone is formed and the cartilage part ially resorbed Even small remnants of cartilage temporarily preserved from resorption and completely isolated in the bone marrow cavity appear intensely radioactive in autoradio graphs made from 5 to 10 days after ^{35}S administration

This behaviour is apparently peculiar to the anlage of long bones only The results of long and short term experiments respectively are totally different in the ossifying cartilage of short bones, e g, in vertebrae In the latter segments, syn thesis of chondroitin sulphate seems to continue also when the

case the penetration of fibrovascular buds represents the phase which immediately precedes the structural changes in cartil age preparatory to endochondral ossification, namely, cell hypertrophy and matrix calcification (2) The milieu in which cartilage differentiates and grows was modified in the scleral cartilage of the chick embryo by determining a collapse of the eye anlage at the third day of incubation, that is, four days before the beginning of scleral cartilage differentiation In the absence of the mechanical stresses which normally depend on the expansive growth of the eyeball, the scleral cartilage of the diminutive bulb grows 3-5 times thicker than the sclera of the contralateral eye, whose development is not impaired (Weiss and Amprino, 1941) It seems that the greater the thickness of a cartilaginous plate, the poorer its metabolism, because of the changes in the proportions between volume and surface, which might seem unfavourable for nutritional exchanges It was shown, instead, that under these conditions (Amprino and Pansa, 1955), the uptake of radiosulphur per unit volume of tissue, photometrically analysed in auto radiographs, is identical in the sclera of the normal and of the rudimentary bulb, also the mitotic rate of chondrocytes and the amount of intercellular matrix for given volumes of tissue proved equal in the sclera of the two eyes

The question arises as to whether renewal of chondroitin sulphate represents a widespread process in cartilage or is limited to the regions in which endochondral ossification is in progress The ready uptake of radiosulphate even by the apparently static cartilage of adults, as shown by Bostrom and Mansson (1953) Dziewiatkowski (1954) and by Pelc and Glucksmann (1955), might favour the assumption that the sulphate is utilized in the renewal of chondroitin sulphate It must be stressed, however, that even after growth of the body has ceased, that is when gross changes in the size of the cartilaginous pieces no longer occur, structural modifications still take place in cartilage in man at least (Amprino and Bairati 1933) and in other big mammals they extend beyond adult into senile age The progressive transformation of the

further evidence in favour of the suggestion that chondroitin sulphate may be utilized in the process of calcification of cartilage or in the laying down of bone, or else that its removal from cartilage is indispensable for the process of fixation of calcium salts on the cartilage matrix

As for the mechanism of liberation of chondroitin sulphate from cartilage, it was suggested that it might be broken down by the shift in pH or by the action of enzymes from the primitive bone marrow (Sylven, 1948) An alkaline medium favourable for the activation of phosphatase would thus be established, which is in agreement with Dziewiatkowski's suggestion (1951) that there might be some "relationship between the metabolism of sulphate sulphur and phosphorus in loci where ossification is progressing"

It can be maintained, in general, that variations in radio sulphate uptake in cartilage always accompany structural changes of the cartilage (Amprino, 1955) Though the structural changes may seem at first different from case to case, they nearly always have at least one feature in common, they depend upon or are accompanied by synthesis of chondroitin sulphate, which is added to the pre existing chondroitin sulphate or replaces it

The rate of sulphomucopolysaccharide synthesis and turn over in cartilage seems to depend exclusively—or nearly so—on intrinsic factors, namely on the activity proper of the chondrocytes within certain limits, the environmental conditions do not seem to influence the ability of cartilage cells to lay down matrix Two examples are given in support of this assumption (1) The penetration of vascular channels into the epiphyseal cartilage of the long bones in the chick embryo does not appear to determine changes in sulphate uptake in areas which on account of their proximity to the vessels, might be expected to find a more readily available supply of nutritional elements in comparison with zones which are at a greater distance from blood vessels This does not hold true for the anlage of the cartilaginous epiphyses of mammal long bones, but it must be recalled that in the latter

the formation of the osseous matrix Such utilization should not imply a direct transfer of chondroitin sulphate as such from cartilage to bone matrix but rather an incorporation in the forming bone of materials which are hydrolysed and set free from cartilage before or during the breakdown of the cartilage Therefore, the osteoblasts must be operative The ability of the osteoblast to lay down the organic matrix of bone tissue should no longer be a matter for discussion, although its mechanism is still obscure On the other hand, the amount of sulphomucopolysaccharides synthesized in the formation of bone matrix would be extremely small in comparison with cartilage, such a quantitative difference seems to depend, at least in part, on the basic differences in the growth processes undergone by the two tissues from the moment they are laid down In fact, no substantial changes in the organic constituents of the intercellular matter of bone occur but only a progressive increment of the mineral phase, a loss of water and perhaps some modifications in the degree of polymerization of the mucoproteins that is, no inner growth processes take place in bone while they occur to a large extent in cartilage

Labelled sulphate administered *in vivo* gets fixed also on bone tissue (Dziewiatkowski, 1952 Davies and Young, 1954, Belanger, 1954 Engfeldt, Engström and Bostrom, 1954 Amprino, 1955) Engfeldt and Hjertquist (1954) have shown that fixation of ^{35}S sulphate occurs also in bone slides treated *in vitro* with the radio isotope, in this case the fixation would depend on a heteroionic exchange between ^{35}S and some elements of the bone minerals

Of some interest in connection with the uptake of ^{35}S in bone are the observations of Vincent (1954) He showed that sulphate administered *in vivo* during the structural reconstruction of the compact bone gets fixed on the layers of recently laid down bone tissue which is not yet mineralized and is still orthochromatic It is apparent, therefore, that the sulphate is incorporated at first in the organic matrix before calcification begins a secondary fixation on the mineral

chondrocytes into matrix ("Verdämmerung", according to Schaffer, 1930) is but one of the structural changes which probably imply new formation of matrix, that is, synthesis of new chondroitin sulphate However, the difference between the amount of radiosulphate incorporated in cartilage per unit volume of tissue during a given time in young growing and in adult individuals respectively is very remarkable (Dziewiatkowski, 1954, Amprino, 1955)

In conclusion, although a renewal of chondroitin sulphate is likely to occur, at least in some regions of cartilage, through hydrolysis of the pre existing sulphomucopolysaccharide and synthesis of new molecules of the same kind, this process might well be more limited than is generally assumed It seems probable that the greater part of the chondroitin sulphate which is progressively synthesized does not replace the pre existing one but is added to it This conclusion seems to be in agreement with the histological data, which show that the inner growth processes in cartilage may continue throughout adult life, though at a much lower rate than during the period of growth proper of the body

The fixation of ^{35}S is in general distinctly lower in the fibrous and fibrocartilaginous tissue than in hyaline cartilage In some regions, however, the reverse may be true, for instance in the case of the intervertebral disc in the albino rat Such differences seem to depend on a different rate of synthesis of chondroitin sulphate in the two kinds of cartilage Under some conditions the uptake of ^{35}S is higher in fibrous than in hyaline cartilage In fact when the intensity of the inner growth of hyaline cartilage decreases, the sulphate uptake may become extremely poor if at the same time a neighbouring region of fibrocartilage undergoes a relatively rapid maturation, its uptake of radiosulphate may be temporarily conspicuous Needless to say, variations in the basophilia and metachromasia of fibrocartilage, and in its radioactivity, seem to run parallel with one another

In regard to endochondral ossification, it has been suggested that the chondroitin sulphate of cartilage may be utilized in

whilst the bone tissue, which obviously was being laid down at the beginning of ^{35}S treatment, is distinctly radioactive The radioactivity seems to decrease, but not considerably, after decalcification Differences in the intensity of the autograph are apparent in the various layers of trabecular bone tissue which have been laid down in successive periods of time since the administration of radiosulphur, they seem to depend chiefly on the gradual decrease in the amount of ^{35}S sulphate circulating in the blood The older trabeculae are more highly radioactive, those formed 6 to 7 days after ^{35}S administration show almost no activity

The obvious conclusion seems to be that both in bone and in cartilage formation, sulphate is introduced at first into the cells which participate in the production of matrix The fact that in cartilage the transfer of radiosulphate from the cells to the matrix occurs apparently more rapidly than in bone tissue, might depend on the different amount of matrix which is laid down per unit time by the osteoblasts and the chondrocytes respectively

Evidence has come from *in vitro* experiments (Engfeldt and Hjertquist, 1954) that sulphate is introduced also into the mineral fraction of bone tissue (cf page 97) It is still doubtful whether the same mechanism may account for the sulphate incorporated in bone *in vivo* According to Engfeldt and Hjertquist (1954), as much as one half of the total sulphate incorporated in bone is in the mineral fraction

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phase through exchange processes would eventually take place as a further step

Dziewiatkowski (1951) had pointed out that in rapidly grow ing rats, after ^{35}S sulphate administration, whenever there was evidence of localization of sulphur in the diaphysis it was primarily in the periosteum No disagreement seems to exist between Dziewiatkowski's and Vincent's data In fact, owing to the limited resolution of contrast autoradiographs and the thinness of the endosteal lining of the vascular channels of the diaphysis in the adult in regions in which the laying down of bone tissue occurs, the radioactivity of the cellular layer could hardly be noticed On the other hand, the periosteum of the diaphysis of rapidly growing rodents is a thick structure and its radioactivity might well disguise a radioactivity which depended on the adjacent and thin layer of recently laid down bone tissue proper Furthermore, Engfeldt, Engstrom and Bostrom (1954) maintain that in the compact bone of the diaphysis in one growing dog, the lining of the Haversian spaces proved radioactive after administration of ^{35}S sulphate

Autoradiographic analysis of the anlage of long bones in the chick embryo treated with radiosulphur has thrown some light on this debatable problem Short and long term experi ments have proved complementary to each other A few hours e g , from 2 to 24 hours, after radiosulphate treatment, radio activity is apparent only at the level of the periosteum and of *the cellular lining of the perichondral bone trabeculae, i e*, where laying down of bone tissue occurs, no radioactivity of the previously formed bone tissue is autoradiographically detectable under these conditions From 24 to 32 hours after sulphate administration, radioactivity seems to appear also in the recently laid down subperiosteal layer of bone tissue of the diaphysis, but the resolution of the emulsion is too low to allow of a clear cut discrimination being made between the activity of the osteoblastic lining and that of the subjacent thin layer of recently formed bone tissue From 5 to 10 days after sulphate administration, the activity of the periosteum and endosteum is barely visible or not appreciable at all,

have now got a rather arbitrary darkening of a film or another technique but we want to make this more quantitative Can this be done or indeed have some of our contributors already done it?

Dent Yes that is a very important point

Bélanger As for the distribution of minerals in the bone, this has actually been done by Prof Leblond and co workers (Leblond C P Wilkinson G W Bélanger, L F and Robichon, J (1952), *Amer J Anat*, 86, 289) He has actually cut out pieces of bone from various areas and measured the specific activity It corresponds exactly with the degree of darkening of the photographic emulsion

Randall Is this in developing tissue or in adult tissue?

Bélanger This is in the tibia and I believe also in the humerus of 50 g rats

Follis I can answer Prof Randall s question too We have done it on a chemical basis in developing cartilage You can make slices at various levels and analyse them chemically for Ca P carbonate phosphatase and probably you can do it for S too

Armstrong I think Prof Randall was thinking of something much more refined and Prof Engström may be able to comment on this point

Engström I think the problem of doing quantitative radioautography is very intriguing and difficult It is almost impossible except in some specialized cases with liquid emulsions

Armstrong But you would be satisfied with slices of tissue Prof Randall?

Randall Provided they are thin enough and you can plot curves from your results and so on I was thinking that it would be very interesting to follow up Prof Amprino s work with the chick embryo in a more quantitative way although the amounts of tissue might be rather small It seems to me too that one has the opportunity by that means to use several very important elements we have got Ca and P and S all of which we can follow in similar experiments

Neuberger Randall has made quite an important point because what one measures here is actually the amount of radioactive S in some form possibly in the form of polysaccharide S but in order to draw a quantitative conclusion in terms of turnover one must know the total amount of S present If there is a lot of sulphate in that particular tissue fraction you may get a lot of blackening without having a very high specific activity On the other hand if there was very little it would indicate a very high specific activity In other words any quantitative interpretation of all this in terms of turnover depends really on accurate knowledge of the total amount of the element which one investigates

Nordin It depends on what chemical form the element is in

Meyer I think Profs Amprino and Bélanger must reinterpret their data as being on the synthesis of sulphated polysaccharides One would like to know if the sulphate is all in the form of carbohydrate or for example is there phenyl sulphate especially phenyl sulphate bound to protein particularly in the early stages? I believe it might be possible by using phenyl sulphatase to show in the same way as Bélanger has

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DISCUSSION

Nassim Could it be that when you say that the cartilage cells are secreting some substance they are in point of fact in the process of being disintegrated?

Amprino Well I just used the word that Prof Bélanger writes in one of his papers when he says that chondrocytes excrete chondroitin sulphate into the matrix

Nassim That is referring to increased vascularity where in point of fact I would think the cartilage cells were being disintegrated

Amprino In most instances the cells are not disintegrating at all because they belong to a part of the cartilage which is still growing that is where cells are dividing and matrix is being laid down

Follis This occurs where they are still living and they will not be disintegrating in the sense of dying and being invaded for quite some time

Bélanger Yes I think I have already explained the time factor We see this passage much earlier than we actually see the radioactive material reaching the area of disintegration

Randall I would like to ask a simple general question In three papers to day we had very elegant pictures of how these various elements have been taken up into various tissues and in various parts of the tissues Is the next kind of analysis possible that is pictorial analysis? If one could now knowing this picture as we have seen it to day in several tissues analyse these tissues further dissect out particular portions and say that at such and such a stage of development there is so much S in this particular part then we would have a very much more complete picture in quantitative terms than we have at present We

IN VITRO UPTAKE AND EXCHANGE OF BONE CITRATE*

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The discovery of a high concentration of citrate in the skeleton by Dickens (1941) necessitated revisions in the concept of the constitution of the bone mineral (Armstrong, 1950) and directed attention to the possibility that citrate plays a rôle in the calcification process or in the localization of the mineral deposit Some of the items of evidence that citrate may be concerned in bone formation and metabolism are (a) the high amount of citrate in calcified tissues—0 95 per cent in rabbit cortical bone (Dixon and Perkins 1952), 1 6 per cent in dry fat and protein free ox bone (Dickens 1941) and 0 8 per cent in human dentin (Free, 1943), (b) the con current elevations of serum calcium and citrate levels under the influence of parathormone (Alwall, 1944) and of vitamin D (Freeman and Chang, 1950), (c) the formation of citrate containing precipitates with compositions similar to those of bone and dentin from solutions of inorganic and citrate ions with concentrations like those of a serum ultrafiltrate (Kuyper, 1945) (d) the healing of rickets in rats (Shohl, 1937) and in children (Shohl and Butler, 1939 Harrison 1953, Harrison and Harrison, 1952) produced by the oral administration of citric acid sodium citrate solutions (e) the increase in citrate content of blood, kidney heart small intestine and bone resulting from physiological doses of vitamin D given to rats receiving normal or rachitogenic rations (Steenbock and Bellin 1953) and (f) the recognition of the central importance

* Supported by a grant from the Research Grants Division of the U S Public Health Service

done with hyaluronidase whether, particularly in the early stages there is non carbohydrate sulphate formed and what is the role of the large distribution and the large concentration of phenyl sulphatase in the tissues

Bélanger Do you think Prof Meyer that these substances would be retained in histological sections?

Meyer If tyrosine or any other hydroxyamino acid is tied up with the structure of the protein of course it would One knows that the only function of vitamin C in the mammal is the synthesis of tyrosine and there is some histological evidence that there is polysaccharide produced but it is not sulphated

Kodicek With regard to vitamin C I cannot say anything about bones, but in normal granulation tissue a dose of radioactive sulphate is found mainly in the mucopolysaccharide fraction as isolated by paper chromatography and it is a sulphated mucopolysaccharide which has the same or similar mobility as chondroitin sulphate In vitamin C deficiency one finds a decrease in sulphate incorporation but there is still as much mucopolysaccharide present or more than in normal granulation tissue But since so much sulphate or almost all the sulphate is present as mucopolysaccharide sulphate, there is no space for phenyl sulphate in granulation tissue

output of citrate was markedly elevated by the administration of sodium bicarbonate The continuous replenishment of citrate in the body fluids from the metabolic processes in soft tissues can be expected easily to provide an amount of citrate to supply the needs of citrate excretion and to maintain the skeletal citrate constant under these conditions Also, the results of Beaulieu and Dallemagne (1951) who reported no increase of citrate content of bones in a small group of animals poisoned with fluoracetate can be interpreted to indicate that an increment of bone citrate, produced by elevated levels of circulating citrate, would not be discoverable, in the presence of the existing bone citrate, with the number of animals used

One prominent concept (Hendricks and Hill, 1950, 1951, Hendricks, 1952) of the nature of the bone mineral describes this substance as minute crystals of hydroxyapatite with several of the ions of extracellular fluid, e g sodium, potassium, carbonate and citrate adsorbed on the crystal surfaces If the considerations of the adventitious presence of bone citrate and of its crystal surface location are correct, the citrate content of powdered bone should be subject to change by exposure to solutions of varied composition in *in vitro* experiments The results of such experiments are the basis of this report

Methods

Three materials were used (1) dry, fat free bovine cortical bone powdered to pass a 60 mesh sieve, (2) the dry, nitrogen-free residue of (1) prepared by boiling the original fat free bone in 3 per cent KOH in ethylene glycol (referred to below as "KOH glycol residue") and (3) synthetic hydroxyapatite prepared by the method of Larson (1935) The solutions with which the above solids were incubated had an initial pH of 7 4 In those solutions which contained phosphate this pH was established by adjusting the ratio of dibasic and monobasic phosphate ions thus giving a buffered solution The citrate solutions which contained no phosphate, referred to below and in the Figures as "unbuffered", were adjusted, before

of citric acid in the final common pathway of oxidative metabolism (Krebs cycle)

Dixon and Perkins (1952) made assays for three of the Krebs cycle enzymes in bone which are involved in the formation and removal of citric acid, namely, citrogenase, aconitase and *iso*citric dehydrogenase The activities of these enzymes in bone were considerably lower than in kidney and liver However, in bone the enzyme activities were higher in the more active regions, the metaphyses and epiphyseal cartilage, than in the shaft cortex Also, the citrogenase activity (the enzyme concerned in the *formation of citric acid from acetyl coenzyme* A and oxaloacetic acid) of bone was much greater than that of *iso*citric dehydrogenase Since the latter enzyme can be taken to serve the rôle of removal of citrate it appears that a mechanism exists in bone for the net production of citric acid These considerations lead to the suggestion (Dixon and Perkins, 1952) that the citrate of bone mineral is that which is formed in the bone cells and is co precipitated with the bone mineral during the calcification process

An alternate possibility as the cause of the occurrence of citrate in bone has to be considered, namely, that some and perhaps the major part, of skeletal mineral citrate is present merely adventitiously owing to the constant presence in body fluids of citrate derived from non skeletal tissues Examples of accidental occurrence of ions in bone mineral are fluoride, lead and radium It is possible that citrate, except for its normal presence in body fluids (2–5 mg per 100 ml plasma), is no more characteristic or essential to bone mineral than is fluoride or radium The hypothesis of the non skeletal origin of bone citrate finds its first evidence in the work of Kuyper (1945), mentioned above, which demonstrated that the presence of citrate ion in simulated plasma ultrafiltrates allowed the co precipitation of citrate with deposits formed *in vitro*, of a material resembling bone mineral in composition

The hypothesis of non skeletal origin of skeletal citrate is not contradicted by the work of Class and Smith (1943) who found no alteration of skeletal citrate in rats whose urinary

whereas the original citrate content of the dry, fat free bone was 0 856 per cent The comparatively low uptake of citrate by KOH glycol residue of bone, which never exceeded more than about one third that of dry, fat free bone, is possibly due to a larger particle size of the extracted bone and, perhaps also to the presence in the same substance of more perfect crystals with fewer surface charges The shape of the curve for the KOH glycol residue of bone indicates that this material at first took up citrate from the solution, but, after 60 minutes of

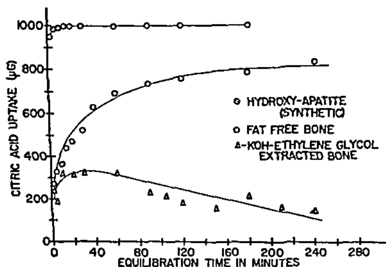


Fig 1 *In vitro* uptake of citrate by hydroxyapatite dry fat free bone and KOH glycol residue of bone with time

incubation, lost citrate to the solution The observations were repeatedly confirmed This finding has no explanation beyond the possibility that the crystals of this material underwent revisions of structure when exposed to the aqueous solution with loss of surface charges

It is a necessary consequence of the hypothesis of adsorption of citrate ions at sites of positively charged discontinuities on the surface of hydroxyapatite crystals in the bone salt (Hendricks 1952) that other negatively charged ions, e g phos phate should also be able to occupy these positions and thus to compete with citrate for the same locations in the bone

dilution to final volume, to the desired pH by titration with 0 1 N NaOH The pH of the solutions remained constant within 0 1 pH unit during the experiments

Two hundred mg amounts of bone, KOH glycol residue, or of hydroxyapatite were transferred to tared round bottomed centrifuge tubes and the tubes were placed in a water bath at 37 5° C, 5 ml of the test solution warmed to the same temperature were added to the tubes and the incubation was continued for the desired time Continuous agitation of the solution and solid phase was secured by passing a stream of nitrogen gas through the suspension in the tubes At the end of the incubation period the tubes and contents were quickly weighed (to give, with the specific gravity of the solution, the volume of the supernatant fluid) and the solid phase was rapidly separated from the supernatant fluid by centrifugation and decantation The citrate concentration of the supernatant fluid was determined by the method of Zipkin and McClure (1949) The difference between the total citrate contents of the original solution and of the final supernatant fluid was taken to be the amount of citrate which was acquired or released by the solid phase

Results and Discussion

Fig 1 presents a comparison of the citric acid uptake by the three solid materials over periods of 2 to 240 minutes from neutralized solutions of citric acid with an original citrate content of 20 mg per 100 ml (1 04 $\times 10^{-3}$ moles per litre as citric acid) The synthetic hydroxyapatite rapidly and nearly completely removed all of the citrate from solution since the total initial citrate content of the solutions was 1000 µg The citrate uptake by the dry, fat free bone increased with time over the first 100–120 minutes of incubation until about 75–80 per cent of the citrate had been removed from the solutions The slower rate and lower degree of citrate uptake by bone than by hydroxyapatite are probably due to a smaller particle size of the hydroxyapatite and to the fact that the latter substance was initially devoid of citrate

indiscriminately by citrate or phosphate since, in the presence of phosphate, no marked increment of citrate uptake was found when the citrate concentration of the solution was increased from 20 to 40 mg per 100 ml

The curve for dry, fat free bone in the absence of phosphate indicates that this material has the capacity to acquire citrate,

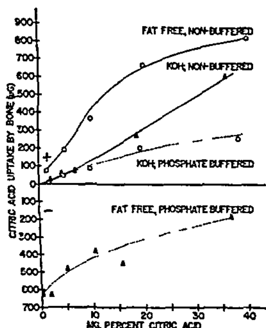


Fig 3 Uptake or loss of citrate by dry fat free bone and KOH glycol residue of bone in the presence or absence of phosphate and at varied initial citrate contents (Phosphate concentration 0 066 molar incubation time 60 minutes points below the horizontal line indicate loss of citrate by bone to the solution)

or at least to maintain its original citrate content, in solutions of citrate concentration in the physiological range (2–5 mg per 100 ml) The loss of citrate by dry, fat free bone at all concentrations of solution citrate shown in the lower part of Fig 3 does not argue against the stability of bone citrate under physiological conditions since the concentration of phosphate employed in these experiments (0 066 molar) was many times

mineral Fig 2 shows that this expectation was realized in the case of phosphate since with increasing concentration of phosphate in the solution lesser amounts of citrate were taken up by the dry, fat free bone from solution of constant initial citrate content When the molar concentration of phosphate buffer exceeded about 0 045 there was an actual displacement of citrate from the bone to the solutions

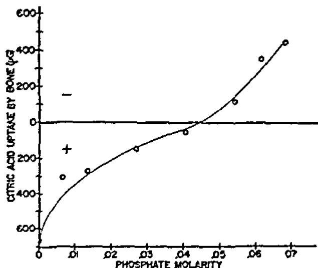


FIG 2 Uptake or loss of citrate by dry fat-free bone in solutions of constant initial citrate content and of varied phosphate content (Initial citrate content of solution 20 mg per 100 ml. incubation time 60 minutes points above the horizontal line indicate citrate lost by bone to solution points below the line indicate citrate taken up by bone from solution.)

Fig 3 presents the results of citrate transfer between solid and liquid phases under conditions of varied initial citrate content and in the presence or absence of phosphate buffer (0 066 mole per litre) The competitive effect of phosphate on citrate uptake by bone was again demonstrated Fig 3 also shows that the same kind of competition was also exerted by phosphate with KOH glycol residue of bone but in this case the effect was of lower magnitude Apparently not all crystal surface locations of KOH glycol bone residue can be occupied

Fluoride is another ion which might be expected to compete with citrate for positions on the crystal surfaces of the bone mineral Table I gives results which show that the presence of 10 mg per cent of NaF in the solutions lowered the amount of citrate taken up by dry, fat free bone from solutions of several initial citrate contents These results are to be attributed to the fluoride ion since KCl in amounts up to 0 066 molar (492 mg per 100 ml) did not alter the uptake of citrate by dry, fat free bone from solutions of 20 mg citrate

Table I

CITRIC ACID—FLUORIDE COMPETITION

Concentration	*µg Citric Acid Taken up by Bone*	
mg % Citric Acid	Citric Acid + 10 mg % NaF	Citric Acid Alone
5	132	158
7 5	172	213
10	295	360
15	409	502
20	490	665
40	788	821

Time 60 minutes

per 100 ml initial citrate concentration The failure of potassium and chloride ions to interfere with citrate uptake by bone was predictable on the basis of theory since bone mineral contains only a small amount of potassium and is devoid of structurally combined chloride

Table II gives results which show that the presence of the ions of the dibasic acids succinic and glutaric acids in the solutions did not interfere with the uptake of citrate by dry, fat free bone However, the ions of the tribasic acid aconitic acid, which in structural formula is related to citric acid, appear, in the concentrations used, to have exerted some degree of competition with citrate ions for uptake by bone

higher than the normal range of phosphate concentration in body fluids (1 0 to 2 0 $\times 10^{-3}$ molar)

If phosphate and citrate compete for the same positions on the crystal surfaces of bone mineral the uptake of citrate by bone should displace phosphate and introduce this ion into the solutions This expected result was obtained as is shown in Fig 4 in which the total phosphorus (as phosphate) per

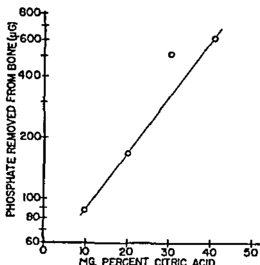


FIG 4 Phosphate removed from dry fat free bone by solutions of citrate (15 ml Volumes of citrate solution incubated for 60 minutes with 600 mg bone)

15 ml of solutions after 60 minutes of equilibration are plotted against their original citrate contents These results do not appear to be due entirely to solution of the whole bone salt complex in the citrate solutions since, although the solutions contained calcium the amount of calcium so contained did not increase with increasing concentration of solution citrate For example the supernatant fluids from the 10 and 40 mg citrate per 100 ml experiments contained respectively 0 30 and 0 26 mg total calcium Preliminary experiments have also shown that treatment of dry, fat free bone with citrate solutions (30 mg citrate per 100 ml) displaces about 0 7 per cent of the bone inorganic carbonate

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DISCUSSION

Fanconi You work here with dead bone but in living bone we have no competition between phosphate and citrate In rickets both phosphate and citrate levels are very low in the blood and in hypervitaminosis D the blood citrate level is raised but the phosphate remains nearly the same Perhaps your experiments in dead bone are different to experiments in living bone

Armstrong Prof Fanconi I do not believe that the results of our experiments are in actual disagreement with the particulars which you cite in reference to rickets In healing rickets there is an increment of bone mineral which requires that phosphate be deposited as a part of newly formed bone mineral This is then a situation of net increase of bone phosphate and of citrate I was interested in what happens in the revision of the detailed composition of the bone mineral once it is formed I would also point out again that in order to demonstrate the competition between phosphate and citrate I used very high concentrations of phosphate This fact does not argue against my thesis that positions on the bone mineral surface may be occupied alternately by citrate and phosphate so that I believe that you and I are not in competition as phosphate and citrate are !

Dixon Some time ago we did some work which showed that due to the fact that there was this very great turnover of citrate in the tissues measurement of citrate content at a particular time would not necessarily indicate the movements of other substances which the citrate metabolism was inducing that is although you might have a comparatively low amount of citrate in that particular tissue at the time it would not mean that it had not in its turnover moved quite a lot of Ca out of a substance and then had been metabolized in the usual way

Table II

COMPETITION STUDIES CITRIC ACID WITH ORGANIC ACIDS

Data given as per cent of citric acid removed from incubating solutions by fat free bone

Organic Acid	20 mg per cent Citrate		10 mg per cent Citrate	
	20 mg per cent Organic Acid with Citrate	Citrate alone	30 mg per cent Organic Acid with Citrate	Citrate alone
Succinic	65 8 ± 1 1	66 8 ± 0 7	71 6 ± 0 9	74 8 ± 0 9
Glutaric	64 0 ± 0 8	65 9 ± 1 1	77 0 ± 1 7	77 1 ± 1 0
Aconitic	61 8 ± 1 0	69 3 ± 0 7	62 9 ± 0 8	74 8 ± 0 8

Time 60 minutes

Preliminary experiments to measure the *in vitro* exchange of bone citrate have been conducted Dry, fat free bone samples were equilibrated with unlabelled citrate solution (20 mg per cent) for 4 hours Citrate labelled with ^{14}C was then added to the solutions without affecting their citrate concentrations The initial 4 hour equilibration with unlabelled citrate was used to allow the net uptake of citrate from the solutions to become constant (Fig 1) The relations of the specific activities of the citrate in the solutions and bone after one hour equilibration following the introduction of the labelled citrate showed that 38 per cent of the total bone citrate had been exchanged This result would require that all of the citrate taken up in the initial period and 17 per cent of the original bone citrate be renewed

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Dixon I suppose this crystalline residue is a very good absorbent for many organic substances for example if you could measure the uptake of glucose by the crystals you should find that some was taken up

Armstrong I think only charged ions would be effectively taken up by bone mineral

Dixon Is it not analogous to animal charcoal in some way?

Randall This has been used as a column

Dixon Does the bone powder act merely as an absorber?

Dent It would not be acting as an ion exchange resin You can separate sugars on some columns but that is old fashioned chromatography it is not dependent on ionic forces

Armstrong Bone has been used as an ion exchange column to remove fluoride from drinking water The water is filtered through bone mineral and the fluoride is removed The material can be regenerated for re use by cooking the bone mineral with NaOH

Neuberger It would be interesting to vary the structure of the compounds for instance to compare tricarballylic acid, which has not got a hydroxyl group with citric acid and see whether it makes a difference It has not got quite the symmetry of citric acid, but one feels that the hydroxyl group is probably involved in addition to the charge

Blaxter I was interested in your observation on the fluoride content of the cow bone The concentrations suggest that there must have been complete penetration of the lattice and therefore there must have been almost complete recrystallization of the whole bone

Armstrong I expect so

Perkins Prof Armstrong you found that about 4 mg per cent of citrate was taken up by your 200 mg of dry fat free bone as compared with 0 856 per cent citrate that was there to start with It does not really represent a very large uptake compared with the amount that was there I wondered whether you were really getting a big increase from your citrate but apparently you were not

Armstrong Since the original bone contained 0 856 per cent citrate the amount of citrate in 200 mg of bone was 1 7 mg With an uptake of 0 8 mg of citrate there was an increment of 49 per cent in the citrate content of the bone Furthermore I am sure we would have got much more taken up if we had continuously replenished the citrate in the solution

Randall In your experiments did you try to find out if the rate process was substantially different or whether only a constant was coming into it?

Armstrong I have no evidence on this point as yet

Randall If you know you are dealing with two ions each of doubly negative charge then they ought to behave similarly unless you have got in one case a triply charged ion and in the other a doubly charged ion

Armstrong We certainly had no triply charged ion

Randall Of course the size of the ion will come into it as well

Perkins That only applies surely to the phosphate Citrate at pH 7 4 should all be completely ionized

Armstrong made a point that there was not much more citrate than other ions in bone and that the citrate was perhaps adventitious but I think bone has 50–100 times more citrate than most other tissues have and you could hardly call a content of that nature adventitious Armstrong used KOH glycol extracted bone and found this curious falling off of the citrate uptake at the end of 2 hours Is that in some way due to the fact that KOH makes the bone alkaline and possibly small amounts of alkali are given up to the solution and affect the ionic strength or H ion concentration? Have you determined the pH before and after? We were familiar with this idea of competition of citric acid with phosphate for Ca and we did experiments where citric acid was added to solutions in which calcium phosphate was precipitated Kuyper showed the solubilizing effect of citric acid on co precipitation and I have always thought that this was just because the citrate solubilized some of the Ca Mg has the same effect in solubilizing calcium phosphate precipitates as magnesium phosphate is much more water soluble than calcium phosphate

I am very surprised at the effect of KF I have always thought that fluoride would form very insoluble CaF_2 which is much more insoluble than calcium phosphate and by some displacement form a layer of this very insoluble CaF_2 which would account for the inhibition of the citrate uptake in the same way Why KF does not work in this way, I do not really see

Armstrong I return again to my conviction that citrate does occur in the bone mineral adventitiously I am fully aware of the fact that bone mineral contains high amounts of citrate Dickens analyses for example calculated to the protein free state show as much as 1 6 per cent citrate in bone mineral That fact alone does not argue against the accidental presence of citrate in bone mineral The affinity of calcium phosphate for citrate and the constant presence of citrate in body fluids account for its occurrence in bone I would point out again the analogous circumstance with regard to the fluoride in bone Fluoride may be present in very high amounts in bone I know of one analysis of a bone from a cow that had been poisoned with fluoride which showed enough fluoride for the bone mineral to be fluorapatite—about 3 56 per cent I submit that this is an obvious case of an adventitious occurrence of a substance in the bone mineral I am like you uncertain as to the interpretation to be put on our results with the glycol ashed bone As far as I can answer your question I will say that this material was simply extracted in the usual way with hot KOH in ethylene glycol washed until the washings were neutral and then dried However I am sure that the K concentration in this material was higher than it was in the original bone

Perkins Have you done any experiments in a series competing the carbonate with citrate?

Armstrong No we have not but yours is a good suggestion The only thing we have done with carbonate is to see whether or not the bone carbonate is displaced when citrate is taken up By setting up a train we could collect CO_2 from the solutions indicating that small amounts of CO_2 were displaced from bone by citrate

THE MAGNESIUM CONTENT OF BONE IN HYPOMAGNESAEMIC DISORDERS OF LIVESTOCK

K L Blaxter

The Hannah Dairy Research Institute Kirkhill Ayr, Scotland

Farm ruminants are prone to a disease characterized by hyperexcitability and associated with a considerable reduction in the serum concentration of Mg The syndrome occurs in mature lactating cows when it is called "lactation tetany" or, more descriptively "grass staggers" It occurs also in growing cattle of both sexes and in young calves in the first few weeks of life Vital statistics are lacking for farm livestock, but the annual incidence in suckling calves has been placed at 5 per cent (Blaxter and Sharman, 1955) while the incidence in cows in some areas may be even greater (Stewart, 1954) It is a serious economic problem to the livestock industry since mortality is high and convalescence often pro tracted The disease has been described in detail and occurs in many countries, treatment consists of intravenous Mg therapy (Dryerre, 1932, Blakemore and Stewart, 1932–3, Sjollema, 1932, Allcroft, 1954) The disease occurs in sheep also, and in horses (Green, Allcroft and Montgomerie 1935) but has not to my knowledge been encountered in pigs Accounts of the occurrence of a comparable syndrome in man have appeared (Hirschfelder 1933) and recently low serum concentrations of Mg have been noted in cases of idiopathic calcinosis in infants This suggests that naturally occurring disorders of Mg metabolism are certainly not limited to ruminant animals

Since members of the symposium may not be familiar with these so called 'hypomagnesaemic disorders" of livestock

Armstrong How many carboxyls are ionized? One or two? I should think two at pH 7 4

Neuberger The last pK is about 6 0, isn t it?

Armstrong The first is 10^{4} and the second is 10^{5} I do not recall the value of the third pK

Neuberger The third one is under 7

Armstrong So you suggest then that I am dealing with citrate 3 minus?

Neuberger Mostly with the trivalent ion

Dixon I have always regarded the calcium citrate complex as not just being a calcium citrate salt but analogous in some way to the copper tartrate which we use in Fehling s solution you get OH coming in and making the complex It is not just Ca_3 (citrate)$_2$ is it? It is the OH binding rather like Cu groups bound onto the hydroxyl groups in tartaric acid What is your concept of the structure of calcium citrate?

Perkins It is usually given as calcium citrate one minus It is rather peculiar that the amount of Ca which came into solution was so small and that it was even lower at the higher citrate concentration than it was at the small one

Armstrong These results are all within the limit of error of the determination 0 3—0 26 mg of Ca

Neuberger It would be quite interesting to see what As does it is so closely related chemically and biologically to phosphate

Meyer It seems to me there are at least two possibilities either that you have an ionic exchange or that you have an ion exchange resin but if this is an ion exchange resin then it is not surprising that there is very little Ca going into solution because you could not know *a priori* what the binding sites are in your resin

Armstrong I think it is a kind of ion exchange since aqueous extracts of dry fat free bone contained vanishingly small amounts of phosphate

Meyer You may have both types which would of course explain the small amount of Ca which is solubilized

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Since members of the symposium may not be familiar with these so called "hypomagnesaemic disorders" of livestock

some attention is given to physiological aspects other than those directly concerned with the metabolism of bone The central importance of the exchange processes involving the bone mineral is, however, dealt with in more detail The information presented is based on studies of Mg metabolism in cattle, the results of which have not yet been published in full To avoid constant reference to our published work, the *papers concerned have been listed and are not specifically* mentioned in the text (Blaxter and Rook, 1953, 1954, 1955, Blaxter, Rook and MacDonald, 1954*a*, 1954*b*, Blaxter and Sharman, 1955, Blaxter, 1955) Most of our studies have been made with young calves in which a Mg deficiency is readily produced by feeding an artificial liquid diet virtually free of Mg (0 4 mg /100 g)

The Mg content of the calf at birth can be predicted from the equation Mg(g) = 0 655W − 3 5, W being the body weight in kg , the standard error of estimate being ± 2 3 The equation was based on the analysis of the complete bodies of calves Some 60 per cent of the total Mg is present in the skeleton and 40 per cent in the soft tissues A calf weighing 40 kg at birth would thus contain some 13 5 g skeletal Mg and 9 g soft tissue Mg The normal accretion of body Mg in growth depends primarily on the growth rates of the soft tissue, notably the muscle mass and the skeleton, and can be followed by determination of the cumulative retention of Mg by metabolic balance methods At the same time determinations of the cumulative retention of N and Ca allow an approximation to be made to the growth rates of the soft tissues and the skeleton In long term growth experiments Mg accretion can, in fact, be estimated from the cumulative retention of Ca and N by making the assumption that the ratio of Ca Mg in bone and the ratio N Mg in muscle remain constant Table I shows the experimental validity of the assumption that in the normal calf given adequate Mg, the ratio Ca Mg in bone is 50 1 and in muscle the N Mg ratio is 140 1 Agreement of the calculated retention with the observed retention over 64 days' experiment was within 1 per cent

Table I

THE CUMULATIVE MG RETENTION OF A NORMAL CALF GIVEN A RATION CONTAINING ADEQUATE MG AND THE RETENTION ESTIMATED FROM SIMULTANEOUS ESTIMATES OF N AND CA RETENTION

Age of calf in days	*Retention of body magnesium up to a particular age*	
	Directly estimated from the balance of intake and excretion (g)	*Indirectly estimated from the simultaneous estimates of N and Ca retention* (g)
0	0 0	0 0
8	0 80	0 58
16	1 34	1 28
24	2 22	1 96
32	3 55	3 07
40	4 28	4 16
48	5 85	5 04
56	7 02	7 17
64	7 82	7 90

The initial Mg content of the body calculated from the body weight was 20 0 g

It is now of interest to apply these approximate factors to a study of experimental Mg depletion In Table II, data are presented for a calf given a diet free of Mg During a period of 48 days a total loss of 3 1 g of body Mg took place In the same period, however, the N retention and Ca retention were positive, that is growth of soft tissues and the skeleton occurred Experiments with a series of deficient calves and of normal ones showed in fact that given equal amounts of food equal amounts of dietary N were retained by both groups until the final few days of life when the grossly tetanic deficient calves stored somewhat less Deficient calves, however, habitually stored slightly more Ca than normal ones given equal amounts of dietary Ca From the Ca and N retentions of the deficient calf in Table II it may be calculated that had the Mg content of the tissues remained unaltered, some

Table II

THE CUMULATIVE LOSSES OF Mg FROM THE BODY OF A CALF GIVEN A Mg LOW RATION AND THE ESTIMATE OF WHAT RETENTION WOULD HAVE OCCURRED HAD Mg RETENTION PARALLELED Ca AND N RETENTION AS IN THE NORMAL ANIMAL

No of days of deficiency	*Observed cumulative Mg retention* A (g)	*Indirectly estimated retention from simultaneous estimates of Ca and N retention* B (g)	*Mg estimated to be incorporated in muscle* C (g)	*Total loss of Mg from the skeleton* (C−A) (g)
0	0 0	0 0	0 0	0 0
8	−0 53	+0 72	0 27	0 80
16	−0 98	+1 42	0 58	1 56
24	−1 70	+2 66	1 22	2 92
32	−2 11	+3 70	1 54	3 65
40	−2 73	+5 13	2 31	5 04
48	−3 10	+6 31	2 90	6 00

6 3 g Mg would have been stored The body deficit of Mg relative to the final body size was thus 9 4 g Further experiments using the same technique showed that body losses of Mg in animals given diets virtually free of Mg were invariably of less importance than the continuing growth of the skeleton and soft tissues in determining the total body Mg deficit The daily loss of Mg from the body when no Mg was given was in fact, rather constant irrespective of growth rate, reflecting a considerable daily loss of Mg in the faeces which presumably represents the unabsorbed part of the continuous inflow of Mg into the digestive tract in glandular secretions

Tissue analysis showed that the composition of soft tissues was not significantly affected in the deficiency (*vide infra*) The body deficit of Mg must therefore have involved depletion of the bone and depletion of the small amounts of Mg present in extracellular fluids The distribution of Mg in the body in the above experiment can be given approximately as follows (Table III)

Table III

THE APPORTIONMENT OF MG LOSSES TO THE SKELETON IN MG DEFICIENCY AND QUANTITATIVE ESTIMATION OF TRANSLOCATION

	Initial quantity based on body weight (g)	*Estimated final quantity had normal retention occurred based on cumulative N and Ca balances* (g)	*Final quantity determined by cumulative balance of Mg intake and excretion* (g)	% *change*
Total body	24.0	30.3	20.9	−31
Skeleton	14.4	17.8	8.4	−56
Soft Tissues	9.6	12.5	12.5*	NIL

* Assuming no depletion of the soft tissue cells

The final concentration of Mg in the bone ash of the calf was 0.32 per cent, the initial concentration as obtained by analysis of a series of calves being placed at 0.6–0.7 per cent. This gives a fall in concentration of about 50 per cent, in close agreement with the 56 per cent estimated from the balances of intake and excretion of N, Ca and Mg. Bone muscle translocation of Mg is placed at 2.9 g—over 50 mg/day. This suggests that in Mg deficiency a complex process involving the skeleton occurs. Mg already present in the bone is removed, part is excreted, and part is transferred to the soft tissues, while, at the same time, the mass of the skeleton is increased by a continuing deposition of Ca and P. Translocation in this context means a gross removal of Mg from bone to soft tissue, not a "turnover" in the sense that the Mg of bone and soft tissue are presumably in a dynamic equilibrium. Translocation in the deficiency may be regarded as indicative of a continuously changing shift of such an equilibrium in response to changes in concentration and energy gradients which alter as muscle growth proceeds.

These findings provide a quantitative measure of the translocation of Mg but are contingent upon demonstration that

even in the acutely deficient animal the Mg content of the soft tissues, comprising 85 per cent of the body mass, is not depressed Direct analysis of muscular tissue from calves for Mg reveals a wide variation in Mg content, from 16 to 26 mg / 100 g This largely reflects the composite nature of the tissue, that is "dilution" of the muscle cells by fat cells and by interstitial oedema fluid, both very low in Mg content By simultaneous determinations of Na, K and Mg in samples of muscle from 8 grossly deficient calves and 8 normal ones, relative intracellular concentrations of Mg can, however, be obtained The results are given in Table IV, and show that

Table IV

THE MG/K RATIO IN CALF MUSCLE AND MG CONTENT OF MUSCLE CONTAINING 400 MG K/100 G MEAN VALUES FOR 2 MUSCLES FROM 8 CALVES IN EACH GROUP

Animal	*Ratio K/Mg*	*Estimated Mg content of muscle containing 400 mg K (mg /100 g)*
Mg deficient	0 0576	23 05
Normal	0 0585	23 41
Standard error of mean differences	±0 0017	±0 66

the ratio of K which is predominantly intracellular in distribution, to Mg did not differ between the two groups, and that the Mg content of muscle containing 400 mg K/100 g was only 0 36 mg lower in Mg deficient animals This small discrepancy was quantitatively accounted for by the presence of extracellular fluid measured by the Na concentration in the tissue There is no doubt, therefore, that the intracellular Mg is unaffected by the deficiency, and that transfer of Mg from bone to muscle cell is the normal reaction of the growing animal to a dietary shortage of the element

The constancy of such relatively high levels of cellular Mg raises problems concerning the energy cost of maintaining

the extracellular intracellular Mg concentration gradient In deficient calves this can amount to more than 50 1, that is much larger than the K gradient of about 20 1 It also raises problems concerning the genesis of the hyperirritability of the deficient animals In this regard it might be thought that since Mg participates as activator in many enzyme systems concerned in carbohydrate metabolism and energy transfer, that Mg deprivation would result in abnormalities of intermediary energy metabolism Indeed, at first we thought that the opisthotonus, the locomotor ataxy, the increased O_2 consumption, the cardiac abnormalities and, what appeared at the time to be more significant, the terminal elevation of pyruvic and lactic acid in the blood of acutely deficient calves was referable to an effect of Mg deprivation on thiamin containing enzyme systems in which Mg acts as an activator It was shown, however, that these changes had their origin in the greatly increased involuntary muscular contraction that takes place in the terminal tetanic stages in which an anaerobic dissimilation of pyruvate predominated The fact that a similar syndrome may be produced in normal animals by severe muscular stress, further suggests the terminal signs do not arise as a result of failure of Mg activated intracellular enzymes This conclusion is in complete accord with the results of the tissue analyses These results further suggest that the genesis of tetany lies in the derangement of systems which lie at the interfaces of cells and which are normally activated by ions present in the extracellular fluid Enzymes concerned in synaptic and neuromuscular transmission are particularly suspect since the injection of drugs affecting choline esterases leads to an exacerbation of clinical signs in the calves while evidence was also obtained of a Ca Mg antagonism in grossly deficient animals The work of del Castillo and Engback (1953) makes such an effect a considerable possibility, but the implications have not yet been pursued

The removal of Mg from the bone similarly raises a number of problems concerning both physiological mechanism and kinetics Histological studies of the costochondral junction of

calves dying in convulsions showed that the normal process of calcification was not impaired (see Fig 1 *a* and *b*) This is of interest in view of the importance of phosphomonoesterase I (alkaline phosphatase) in the calcification process, an enzyme activated by Mg ions The optimal concentration of Mg ions necessary for activation of this enzyme is M/200 (Roche, 1950) The concentration of Mg in the serum of the calves in the final stages was M/500, and if the ultrafiltrable portion is taken to be wholly ionized, the Mg ion concentration was approximately M/1000 This might suggest that the serum enzyme would show low activity but we were not able to demonstrate any fall in alkaline phosphatase activity in the serum in the deficiency or any increase in its activity on addition of Mg ions to the serum This probably reflects either activation of the enzyme prior to its release from the cell or the ability of Zn and Mn ions to activate the enzyme in very minute concentrations (M/10000–M/50000) or the similar activating effect of certain amino acids The fact that, histologically, calcification is normal, that Ca retention is slightly enhanced and that the concentration of inorganic P in the serum of the calves is unaffected by the deficiency, suggests that phosphatase activity at the site of mineralization is normal too It is probable, in fact that Mg is concentrated by osteoblasts just as it is by muscle, liver and other body cells certainly the high Mg content of the ash of imperfectly calcified bone suggests that osteoid tissue has a high Mg content No impairment of calcification due to shortage of Mg for phosphatase activation would thus be expected

In sheep deprived of dietary Ca (Martin and Peirce 1934) and indeed in other species demineralization of the skeleton is not general The trabeculae of the cancellous ends of long bones are more affected than the cortical bone of shafts, and the vertebrae, ribs and skull bones more than those of the extremities (Duckworth and Hill, 1953) To find whether the pattern of removal of Mg followed the pattern of overall demineralization the shafts and the ends of ribs were analysed separately As shown in Table V, the Mg content of the bone



Fig 1 (a) The costochondral junction of a Mg deficient calf

[facing page 124

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since the experimentally determined value does not differ significantly from the ratio of the two atomic weights

The process of depletion probably takes place at the surface of the bone crystals by an exchange process, indeed, part of the bone Mg can be removed by suspending powdered bones in dilute solutions of Ca ions It is not known how the recrystallization processes take place when Mg is replaced by Ca on the crystal surface or when Ca is replaced by Mg The Mg ion is smaller (0 78 Å) than the Ca ion (1 09 Å) and some differences in crystal structure might ensue on replacement of the one by the other How far, and, more important at what rate, recrystallization modifies the crystal surface and hence the quantity of Mg available for heteroionic exchange is not known

The nature of the gross kinetics of the exchange process can be inferred from studies of the course of change of serum Mg concentration with time, the simultaneous changes in bone Mg concentration, the rates of loss of Mg ions via the kidney and the gut wall, and the rate of incorporation of Mg in growing soft tissue These processes have to be considered in relation to a continuous exchange of ions ("turnover") throughout the body Clearly they must necessarily be of a high order of complexity Thus the rate of fall of Mg in the serum of deficient calves is not a simple function of time, the logarithmic rate initially being extremely steep, falling slowly over a period of 30–40 days until a concentration of 0 7 mg /1000 ml is reached when subsequently the rate of fall is extremely slow If convulsions and death do not ensue the serum concentration of Mg may fall in a further period of 30–40 days to 0 4–0 5 mg /100 ml Spontaneous small increases in Mg concentration often occur during this period of slow fall They are associated with abolition of clinical signs and thus cannot be ascribed to analytical errors They usually follow digestive disturbances or periods of anorexia Also non fatal tonic clonic convulsions cause an increase in the serum concentration presumably due to the cellular catabolism engendered by the excessive energy expenditure It appears that the animal's appetite for its Mg free ration plays a part in

in depletion of the predominantly cancellated bone of rib or the cortical bone of metatarsal shafts was discernible, even in animals showing a loss of 75 per cent of their bone Mg Slight differences in Fig 1 can be explained by varying degrees of contamination of osteoid tissue Analysis of the terminal coccygeal vertebrae of calves in which low percentages of ash revealed poor calcification gave a higher apparent Mg content of the ash than did the fully calcified metacarpal shaft of the same animal This reflects the high Mg content of the uncalcified matrix, and in better calcified specimens from older depleted calves the discrepancy was not apparent

This suggests that in these infant calves virtually the whole of the skeletal Mg participates in the depletion and translocation process, and that there are no sites at which greater relative depletion occurs This is in agreement with the evidence of $^{14}CO_2$ studies which indicate that in the young animal the amount of bone available for inorganic carbon exchange is close upon 100 per cent of the total while in the adult the proportion is about 50 per cent and probably less (Buchanan and Nakao, 1952) In this regard, it has been noted in our experiments that transfer of cows to Mg low diets gives a more rapid fall in serum Mg than transfer of calves to Mg free diets, and in Allcroft s (1947) field surveys the highest incidence of the natural disease occurred in the older cows, notably those which have calved three or four times These two findings are consonant with the hypothesis that a relative paucity of exchangeable skeletal Mg due to the poor circulation of blood through old established bone, in the adult animal determines the course of the adult disease

An indication of the nature of the association of Mg with the bone mineral was provided by the fact that properly calcified bones low in Mg content usually had a Ca content slightly greater than those containing normal amounts The regression of the Ca content of the bone ash of calves on their Mg content was $-2\ 1 \pm 0\ 5$, entailing a replacement of 1 mg of Mg by $2\ 1 \pm 0\ 5$ mg of Ca In terms of atomic replacement, this means the replacement of one atom of Mg by one of Ca

determining the extracellular Mg concentration and becomes in fact a homeostatic mechanism

The bone Mg in relation to the serum concentration is shown in Fig 3 Removal of the first 200 mg Mg/100 g of bone mineral is associated with a fall in serum Mg from 2 2 to 0 7 mg /100 ml Removal of the second 200 mg Mg/100 g bone ash, however, is associated with a fall in serum Mg from

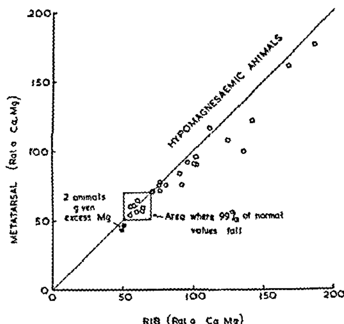


FIG 3 Ca Mg ratio in compact bone of the metatarsal bone shaft and from cancellated bone from the rib Note values for rib slightly higher than those for metatarsal shaft

0 7 to 0 5 mg /100 ml In view of the spontaneous variation in serum Mg concentrations in calves exhibiting a serum Mg concentration below 0 7 mg /100 ml the possibility of predicting the extent of bone depletion by blood analysis does not appear high A value of 0 6 mg /100 ml might reflect a 30 per cent or an 80 per cent depletion of the bone Mg

Renal losses of Mg in deficient calves maintaining serum levels of 0 7 or less are very small—2 mg /day—so small

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DISCUSSION

Dent Can you trace this loss of Mg in the faeces to some secretion?

Blaxter We have not as yet a radioactive tracer in Mg studies but we tried to trace it by giving very large doses of Mg and collecting faeces at intervals It is a high intestinal secretion as judged by the time delays in excretion

Dent You do not know if it is in pancreatic juice?

Blaxter No I do not know yet

Dixon I always thought that Mg deficiency produced very dense bones analogous to marble bones and that the ash content of bone went up enormously

Blaxter We are dealing almost entirelv with infant calves and we have not noticed an enormous increase but there is a slight increase

Dixon What would the relative increase of ash be?

Blaxter Quite small Some of these infant bones are not completely calcified and so it is very difficult to interpret ash percentages I am very chary of doing this

Dixon You mentioned the effect of Mg on serum phosphatase Some years ago we were trying to do urine phosphatase estimations We used to take the urine in bone disease and dialyse it to remove all phosphate and then take the dialysed residue Mg had no activating effect on the urine phosphatase even though it had been dialysed

Blaxter I do not think anybody knows what is the nature of the Mg link

Armstrong Would you consider that your studies demonstrate again that the prime function of the skeleton is as an organ of homeostasis and the secondary function is as an organ of support?

Blaxter Well the very fact that I stand up is some indication that it must have *something to do with support! Certainly as far as Mg is con*cerned it has an incredible capacity for tiding animals over a period of dietary shortage The maximum translocation of Mg that we found is of the order of 5 g in a young animal gaining weight from 40 kg up to 70 kg which is a large amount of Mg to be transferred from the skeleton to soft tissues Nobodv has put this on a quantitative basis before and I think it is an extraordinarily interesting fact that 30–40 per cent of this particular bone mineral can be translocated from bone to muscle quite apart from its net turnover

Armstrong Would you regard bone Mg as an ion of adventitious occurrence?

Blaxter In the way in which you interpret the word adventitious, yes

physiological responses to cold wet weather were postulated in the past as causes of the hypomagnesaemic diseases of cattle and the importance of the relatively slow processes of *repletion and depletion of the crystal surface of the skeleton* had not been considered The importance of maintaining Mg therapy in clinical cases and the prevention of the disease by long continued Mg dosage is, however, now being recognized by veterinary practitioners

What is of some interest at present is that methods of bone biopsy have been developed which allow an assessment of the Mg reserves of animals in which tetany has occurred, thus placing Mg therapy on a more sure basis Analysis of bones from animals which have died suddenly for no apparent reason has enabled the cause of death to be ascertained, and has made possible a separation of certain types of convulsive syndromes of calves thought to be of genetic origin, as well as the encephalopathy of lead poisoning (Allcroft and Blaxter, 1950) from hypomagnesaemic tetany

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Armstrong Would you regard bone Mg as an ion of adventitious occurrence?

Blaxter In the way in which you interpret the word adventitious, yes

Black Has the mother s state of Mg deficiency any bearing on what happens to her calves and if so can you give her something prophylactic ally?

Blaxter Our evidence for that is not very good at the moment but in a large series of analyses of the bones of calves at birth we do find quite a wide variation from approximately 0 54 per cent of Mg in the bone ash up to about 0 7 per cent and this is correlated although not to a very high degree with the serum concentration of Mg of the cow What you do find however is that the milk concentration of Mg of the cow seems to be characteristic of the individual and so some calves receive very low intakes of Mg from their mother s milk and others get quite a reasonable amount

Bélanger What is the actual content of Mg in your experimental diet?

Blaxter It is as low as we can possibly get it which is 0 5 mg /100 g that is about one fiftieth of the normal We cannot get it any lower due to contamination of the casein and analytical reagent grade chemicals which we use

Bélanger What was the condition of the epiphyseal plate?

Blaxter As far as we can tell it was quite normal The photograph was of an animal which had died in convulsions at somewhere between 60 and 70 days

Dallemagne Have you some information about the elimination of K? From the pharmacological point of view, the activity of the central nervous system depends on the ratio $\frac{Ca + Mg}{K + Na}$ If the ratio drops it may be that a loss of K restored normal activity of the central nervous system

Blaxter We have done that experiment in rather a different way We produced K toxicity simultaneously with both normal Mg intakes and sub optimal Mg intakes and we found that we could produce a K toxicity syndrome The K toxicity while it has marked effects on neuro muscular accommodation measured electronically and while it produces oedema and cardiac defects had absolutely no effect on Mg elimination serum Mg concentration the incidence of tetany or the age at death

Lacroix Has a similar study ever been conducted on small sized animals?

Blaxter Yes comparable work was done in 1932 on rats and there rather a different syndrome occurred in which marked vasodilatation was apparent I think a deficiency of pantothenic acid might have been involved in some of that work

Lacroix You mentioned the adult have long bones in the adult been X rayed?

Blaxter No we have not done any X ray studies I do not know whether we would see very much

Lacroix But did you not say that there is a difference between cancellous bone and compact bone? Such a difference might possibly show up on the X ray plate

Blaxter Yes *it may appear there but it is doubtful*

Fig 4 (Blaxter) The heart and great vessels of a calf which died in convulsions when given a Mg low diet

Fig 5 (Blaxter) A section of the pericardial fat of a Mg deficient calf which died in convulsions showing terminal haemorrhage

[*facing page* 13–

Fig 6 (Blaxter) A section of the myocardium of a Mg deficient calf show rupture of a small venule thrombus formation and minimal local reactic

Fig 7 (Blaxter) Congestion of the venae rectae of the kidney in a calf which died in convulsions due to Mg deficiency

Follis We studied the skeletal tissues of McCollum's rats and it is not unexpected for you to find no change we have never seen change until at the very end when the animal stops growing then you see the non specific change I believe kidney changes have been described in rats Have you any evidence of this?

Blaxter No, we have no evidence at all of any gross kidney changes comparable to those seen in rats The only tissue changes we have seen are changes that take place in adipose tissue and are just those that you get in strychnine poisoning Fig 4 shows the typical gross pathological changes in the heart in the convulsive state which are, I think indicative of an agonal death The haemorrhages on the walls of the auricles and ventricles are quite typical Fig 5 which shows a section of the pericardial fat indicates the terminal rupture of blood vessels but no infiltration of white cells at the site of rupture Fig 6 is a section of the wall of the right ventricle Here, there is thrombus formation due to rupture of a small venule and again there are only a few white cells which have localized at the site We find nothing at all in kidney except by congestion of the venae rectae as shown in Fig 7

Follis You see no morphological changes in the myocardial fibres?

Blaxter No

Howard Is this the opposite of hibernation?

Blaxter One could take it as such I suppose One can of course produce coma with excess Mg

Howard Does the hibernating animal's Mg not go up?

Blaxter Yes that goes up very markedly in hibernation

Howard So hibernation is the one thing you know that will upset the homeostatic relationship here Is that provided by the bone too?

Blaxter I do not know that I have never worked with hedgehogs

Dixon We have tried to alter serum Mg in man but with no success I have given doses of $MgSO_4$ and other Mg salts Have you ever done this?

Blaxter Large doses of $MgSO_4$—several pounds to the cow—have a very transitory effect on the serum Mg One can of course put it up by intravenous injection of a Mg salt

Armstrong A student of mine is re investigating the effect of parathormone on the Mg transport He finds a very short lived decrease in plasma Mg after parathyroidectomy of rats The result is statistically valid but whether or not it is physiologically significant I do not know He finds no change in the Mg content of muscle or of red blood cells after parathyroidectomy

Blaxter It is rather interesting that in the deficiency while the serum Mg drops over a period of about 30 days we can begin to detect a decline in the red blood cell Mg in about 80–90 days There is only a very small decline which we interpret as reflecting the Mg concentration at the site of red blood cell formation which in turn determines the concentration of Mg in the new cells being formed

Black You mentioned changes in the serum Mg in infantile calcinosis do you mean the children with hypercalcaemia? And what degree of change did they have?

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Howard Is this the opposite of hibernation?

Blaxter One could take it as such I suppose One can of course produce coma with excess Mg

Howard Does the hibernating animal s Mg not go up?

Blaxter Yes that goes up very markedly in hibernation

Howard So hibernation is the one thing you know that will upset the homeostatic relationship here Is that provided by the bone too?

Blaxter I do not know that I have never worked with hedgehogs

Dixon We have tried to alter serum Mg in man but with no success I have given doses of $MgSO_4$ and other Mg salts Have you ever done this?

Blaxter Large doses of $MgSO_4$—several pounds to the cow—have a very transitory effect on the serum Mg One can of course, put it up by intravenous injection of a Mg salt

Armstrong A student of mine is re investigating the effect of para thormone on the Mg transport He finds a very short lived decrease in plasma Mg after parathyroidectomy of rats The result is statistically valid but whether or not it is physiologically significant I do not know He finds no change in the Mg content of muscle or of red blood cells after parathyroidectomy

Blaxter It is rather interesting that in the deficiency while the serum Mg drops over a period of about 30 days we can begin to detect a decline in the red blood cell Mg in about 80–90 days There is only a very small decline which we interpret as reflecting the Mg concentration at the site of red blood cell formation which in turn determines the concentration of Mg in the new cells being formed

Black You mentioned changes in the serum Mg in infantile calcinosis do you mean the children with hypercalcaemia? And what degree of change did they have?

Follis We studied the skeletal tissues of McCollum's rats and it is not unexpected for you to find no change we have never seen change until at the very end when the animal stops growing then you see the non specific change I believe kidney changes have been described in rats Have you any evidence of this?

Blaxter No, we have no evidence at all of any gross kidney changes comparable to those seen in rats The only tissue changes we have seen are changes that take place in adipose tissue and are just those that you get in strychnine poisoning Fig 4 shows the typical gross pathological changes in the heart in the convulsive state, which are, I think, indicative of an agonal death The haemorrhages on the walls of the auricles and ventricles are quite typical Fig 5, which shows a section of the peri cardial fat indicates the terminal rupture of blood vessels but no infiltra tion of white cells at the site of rupture Fig 6 is a section of the wall of the right ventricle Here there is thrombus formation due to rupture of a small venule and again there are only a few white cells which have localized at the site We find nothing at all in kidney except by conges tion of the venae rectae as shown in Fig 7

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Black You mentioned changes in the serum Mg in infantile calcinosis do you mean the children with hypercalcaemia? And what degree of change did they have?

Blaxter There were only a few figures published on children, but had these figures related to calves I would have expected such animals to be in tetany

Dent Did you not tell me once that the blood Ca rises too?

Blaxter There is a tendency for the blood Ca to rise that is it increases slightly more than one would expect for a compensation for the fall in Mg The increase is about 0 4–0 5 mg /100 ml

Follis How prevalent is this syndrome in other areas?

Blaxter It was first described in Holland where it is extremely prevalent In this country it is one of the major causes of economic loss to dairy farmers In the area in which I have been working in the north of Scotland we think that the incidence is between 5 and 7 per cent It is known in the United States and in Canada and has been reported from Australia There is a great deal of work being done on blood chemistry of the condition in New Zealand It is quite widespread

Dent As far as you are concerned this is true tetany, is it? If you lower the Ca you can get the same type of picture?

Blaxter Yes it is a true tetany There is uncontrolled fibrilation of the muscle and the slope of the accommodation curves drops precipitately

Follis It is not the uncontrolled activity that one sees in rats they run around the cage at a sharp sound

Blaxter We have tried a variety of sounds to produce convulsions and we have not been able to find a sound that will always produce convulsions in animals which are in a state of latent tetany The mere presence of anybody working in the room with them sometimes will produce first of all a paddling motion of the feet and eventually the animal goes down into tetany

Dent Do you know whether it is by alkalosis?

Nordin Yes I wonder how alkalosis induces tetany It is never really explained by the change in ionized Ca as far as one knows Does alkalosis perhaps affect the ionized Mg level very strikingly?

Blaxter We have not done studies on alkalosis yet it is the obvious thing to do next

Nicolaysen What about the ultrafiltrable Ca in the serum?

Blaxter The percentage of the Ca in the serum which is ultrafiltrable is completely unaffected The percentage of Mg which is ultrafiltrable is also unaffected About 60 per cent of the Mg is ultrafiltrable

THE MECHANISM OF NUTRITION IN BONE AND HOW IT AFFECTS ITS STRUCTURE, REPAIR AND FATE ON TRANSPLANTATION

W R Harris and A W Ham

Departments of Surgery and Anatomy University of Toronto

In considering bone it is important to emphasize that it, like all types of connective tissue, consists of *living* cells and *non living* intercellular substances that are made by its cells

Intercellular substances are being considered in detail by other speakers at this conference They are non living organic materials made by cells One ingredient of the intercellular substance of bone, i e collagen, has great tensile strength There are, moreover, other ingredients in the intercellular sub stance of bone that permit it, under normal conditions, to become calcified, that is impregnated with mineral salt, so that it becomes capable of bearing great weight

Bone is only one of several varieties of connective tissue Before considering some of its special features in detail it is helpful to consider some aspects of connective tissue in general

It is in the embryonic forerunner of the connective tissues that the blood vessels of the body all develop Accordingly, after the blood vascular system develops, it is contained in, and carried to all parts of the body by connective tissue Hence, speaking generally, it is through the capillaries that are in connective tissue that all other tissues for example epithelium, are nourished Most of the cells of other tissues and connective tissue itself, however, do not live directly beside capillaries Cells and capillaries are generally separated from one another by varying amounts of intercellular sub- stance Accordingly, there must be some mechanism by which there can be an interchange of nutriments and waste products

between cells and capillaries, such a mechanism involves the passage of materials through the intercellular substance One possible mechanism that could accomplish this end would be that of fluid forming at the arterial ends of capillaries, and then circulating through clefts in the intercellular substance to the venous ends of capillaries and to lymphatic capillaries, where it would be absorbed Free tissue fluid even if it did not circulate could permit diffusion of nutriments from capillaries to cells But, although it is generally agreed that free tissue fluid can exist in tissue spaces under conditions of oedema, it is by no means certain that free tissue fluid exists even in loose areolar tissue under normal conditions (Bensley, 1934, McMaster and Parsons, 1950) However, even with no free tissue fluid, the bound water of the colloidal intercellular substances could serve as a means for diffusion between capillaries and cells That it does so is best illustrated in the instance of hyaline cartilage

Hyaline cartilage is a non vascular tissue Its cells, which lie in lacunae in its intercellular substance, are often separated for considerable distances from the capillaries that lie outside the cartilage Yet the water containing intercellular substances of cartilage permits such an efficient transfer of nutriment that cells deeply disposed in cartilage give every evidence of being able to synthesize protein, both by reproducing themselves, and by forming further intercellular substances

Although hyaline cartilage can persist throughout life in a living uncalcified state, as it does on articular surfaces and in a few other sites, most of the cartilage that develops in foetal life undergoes calcification In this phenomenon calcium salts precipitate into the organic intercellular substance, and this event is associated with the death of the cells that are surrounded by the calcified intercellular substance In ordinary rickets the cartilage at the diaphyseal sides of epiphyseal plates, that ordinarily calcifies, does not do so, probably because of a low CaP product in the surrounding medium Hence the mature cartilage cells that would ordinarily die, as

the intercellular substance about them becomes calcified, continue to live, and since the cartilage continues to grow the epiphyseal plates become greatly thickened in this condition It is therefore a fair inference to suggest that one reason for the calcification of cartilage killing cartilage cells is that the mineral in the intercellular substance prevents the intercellular substance from serving further as an efficient means for diffusion between capillaries and cells

A fundamental difference between cartilage and bone is that in bone there is a means whereby the constituent cells of the tissue can remain alive even though the organic intercellular substance that surrounds them becomes calcified This mechanism is due to the osteoblasts that manufacture bone intercellular substance having long connecting cytoplasmic processes which serve as moulds for future passageways, as the intercellular substance forms around them Accordingly the intercellular substance of bone is permeated by tiny canals, the canaliculi, which permit a means whereby diffusion mechanisms can operate in bone between capillaries and cells even though its intercellular substance becomes densely calcified The canaliculi may contain cytoplasmic processes of bone cells, or if these are withdrawn after bone is formed, as some believe the canaliculi would contain tissue fluid through which diffusion could also occur The basophilic staining reaction of canaliculi and their metachromasia suggest that they are lined with a mucopolysaccharide, this may also assist diffusion phenomena

Although canaliculi permit cells to survive in a calcified intercellular substance the relatively poor system for diffusion that they provide could not be expected to operate over anything more than very short distances Measurement made on a dog's radius (Ham, 1952, 1953) showed that bone cells were generally no further than one tenth of a mm from a capillary so it is probable that the system does not operate very efficiently over greater distances This requires that bone be a relatively vascular tissue so arranged that even in dense bone no cell is generally more than a small fraction of a millimetre

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from a capillary For bone to contain such a good blood supply requires that it be constructed most ingeniously

As many investigators have shown over the last two centuries, by putting metal pegs in a growing shaft, bone unlike cartilage, does not grow interstitially, that is by expand ing The obvious reason for this is that under normal con ditions the intercellular substance of bone becomes calcified soon after it is formed, and calcified bone could no more be expected to undergo expansive growth than reinforced con crete Accordingly, for any bony structure to increase in size requires that new bone be added to a pre existing surface Although it is possible for what is termed coarse fibre bone to form in an area of differentiating osteogenic tissue, as has been emphasized by Baker (1952), for all practical purposes normal bone growth is dependent on new layers of bone being added to pre existing surfaces This must be accomplished in the growth of bones so that no bone cell is removed more than a fraction of a millimetre from a capillary

The deposition of new bone on pre existing bony surfaces is accomplished by the osteogenic cells or osteoblasts that cover and line bony surfaces forming intercellular substances in which they become buried, on those bony surfaces By this means they become differentiated bone cells embedded in the new layer of bone that they have formed However, before covering and lining cells differentiate into bone cells, they divide so as to provide enough covering and lining cells to keep the surface covered

Cancellous bone consists of a scaffolding of narrow bony trabeculae The spaces between the anastomosing trabeculae contain a delicate connective tissue that contains blood vessels From the latter diffusion takes place through the delicate connective tissue and the canaliculi of the bony trabeculae, and so the cells of the trabeculae are nourished If the trabe culae are close enough together and if their covering cells lay down successive layers of bone, the spaces between trabeculae become successively smaller, and often just large enough to comfortably hold the blood vessel The bone, at the same time,

becomes relatively solid or compact in appearance By this mechanism, the laying down of successive layers of new bone by the covering cells of anastomosing trabeculae, cancellous bone can be converted to compact bone Since the blood vessels between trabeculae are preserved no bone cell in the compact bone is too far removed from a capillary to survive

The unit of structure of compact bone is the Haversian system In cross section such a system reveals, usually, a single large capillary, and less often two vessels, one arterial and the other venous, lying in a canal that is lined by osteogenic cells, and surrounded by several layers of bone In such a system no bone cell is more than a small fraction of a millimetre from the central vessel and each cell can therefore survive by diffusion occurring along the canaliculi Such a system can only develop by means of a pre existing tunnel, which contains a blood vessel or vessels, being filled in by successive layers of bone by the osteogenic cells and osteoblasts that line the tunnel For the formation of any Haversian system there must first be a tunnel, and it must contain a blood vessel

Bony tunnels destined to become Haversian system are formed somewhat differently in different parts of the developing skeleton As has already been shown, as cancellous bone becomes transformed to compact bone, the spaces between the anastomosing trabeculae, and which contain blood vessels, serve as the tunnels In growing long bones with flared extremities, tunnels left by breaking down cartilage columns in the periphery of the epiphyseal plates on its diaphyseal side may serve to permit Haversian systems to develop, these systems will be characterized by having cartilage remnants around them to serve as their interstitial lamellae In the growth in width of shafts of long bones, tunnels are formed on the exteriors of the shafts by means of the peaks of adjacent longitudinal ridges, because of osteoblastic activity, becoming fused so as to cover the grooves that lie between the adjacent ridges These longitudinal grooves which thus become tunnels are lined by osteogenic cells and osteoblasts of the periosteum

and contain periosteal vessels The filling in of such tunnels add Haversian systems to the exterior of the shaft Another way that tunnels, destined to be Haversian systems, can be formed is by grooves being eroded on flat bony surfaces with osteoblastic activity subsequently joining the two lips of the grooves

As has already been noted, Haversian vessels are usually single Each single vessel provides nourishment for the bone cells embedded in the lamellae of a system When a bone is broken, circulation stops in these single vessels, on each side of the line of fracture to some point where the vessel anastomoses with a still functioning vessel, and this may be some little distance away When circulation stops, in the segments of Haversian system that are close to the line of fracture, no further nourishment is provided to diffuse along the canaliculi in that segment of the system, and, as a consequence, the bone cells of the affected segment all die Therefore, as a result of the nature of the blood supply of compact bone, and the inefficiency of the canalicular system, a fracture results in the death of a considerable segment of bone on each side of the fracture

The repair of a fracture is brought about chiefly by the proliferation and differentiation of the osteogenic cells and osteoblasts that cover and line bone surfaces Those of the deep layer of the periosteum soon begin to proliferate and gradually form collars that surround each fragment near the fracture line The osteogenic cells that make up these two collars proliferate so vigorously that the collars bulge toward each other and fuse to constitute the external callus In the collars, the osteogenic cells differentiate both into cartilage and bone As was pointed out many years ago (Ham 1930) osteogenic cells can differentiate either into cartilage or bone, depending on whether they differentiate in a non vascular or a vascular environment If the osteogenic cells proliferate rapidly they tend to outgrow the capillaries that would otherwise accompany them, hence rapidly growing masses of osteogenic cells *tend to form cartilage rather than bone* The

cartilage that forms in the repair of fractures is eventually replaced by bone

The internal callus forms from the osteogenic cells that line the interior of the bone and which comprise the endosteum, and also from nearby undifferentiated cells of the marrow, these are exceedingly competent to form bone as well as marrow

Many accounts of fracture healing state that an early and important step in the process is the invasion of the blood clot by granulation tissue We (Ham and Harris, 1955) have recently studied the fate of the blood clot in experimental fractures and have found that so far as the formation of the external callus is concerned, it appears to be of no importance It is not invaded by granulation tissue in the early stages of healing and it appears to be more of an obstacle than anything else to the growth and fusion of the collars of osteogenic tissue that bring about union

An appreciation of the nature of the blood supply of compact bone and the relative inefficiency of the canalicular mechanism, makes it easy to appreciate that the bone cells of transplants cut from compact bone could not be expected to survive Although some of the covering and lining osteogenic cells of a compact bone transplant may, if they are fortuitously located with regard to functioning capillaries, survive transplantation, the bone cells themselves which depend on canaliculi for nourishment, die when circulation stops in the Haversian vessel on which they depend For all practical purposes compact bone transplants should not be regarded as living grafts but as transplants of dead bone Such transplants, however, serve many useful purposes Along and around their sites of contact with the living bone into which they are set, the covering and lining cells of the host bone proliferate, as they do in the repair of a fracture, and the callus tissue that is produced becomes cemented to the transplant and binds it in place Then, slowly, the transplant is eroded and replaced with new bone

It might be thought that transplants of cancellous bone

would survive transplantation providing they were placed in sites where there were functioning capillaries and an abundant supply of tissue fluid But even under favourable conditions such as these the canalicular mechanism does not seem to be efficient enough to permit the survival of more than an occasional bone cell that is close to a surface (Gordon and Ham, 1950) Under such conditions, however, the osteogenic cells that cover the surfaces of cancellous fragments can survive and proliferate and give rise to new bone, which grows towards the functioning capillaries Fragments of cancellous bone can, therefore, be used to set up new centres of osteogenesis under very favourable circumstances

It is sometimes intimated that there is no essential difference between the osteogenic cells that cover and line bone surfaces and the ordinary fibroblasts of connective tissue To see if fibroblasts of muscular tissue would form bone as readily as the covering and lining cells of bone, Ham and Gordon (1952) compared the effects of transplanting thrice frozen and thawed autogenous cancellous bone fragments into muscle with those of transplanting untreated autogenous fragments into a similar site They found that new bone formed from the covering and lining cells of the fragments of untreated bone but did not form from the fibroblasts that grew around the thrice frozen and thawed chips

When thrice frozen and thawed autogenous cancellous bone fragments are planted in muscle they are soon surrounded by granulation tissue The granulation tissue becomes increasingly cellular and so called giant cells then develop in association with the bony fragments In different species and in different sites these display appearances varying from that of typical foreign body giant cells to typical osteoclasts with striated borders The striated borders are only seen on surfaces of these so called cells that abut on bone, and Ham (1952) has suggested that these borders belong to bone rather than to the osteoclasts Striated borders are sometimes seen on bony surfaces where no osteoclast cells are present Since typical osteoclasts can form in granulation tissue where no

bone is forming and where no living osteogenic cells are present, it can be concluded that typical osteoclasts do not necessarily have to arise from osteogenic cells and osteoblasts Furthermore, from such observations as we have made on material of this type, it appears to us that osteoclasts and foreign body giant cells represent more than a fusion of cells, they represent a melting down and shrinkage of areas of connective tissue, which contain both intercellular substances and cells In our opinion osteoclasts are degenerating areas of tissue rather than living cells

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DISCUSSION

Kodicek I would like to mention one substance which has not cropped up during these few days namely vitamin C which has quite an im portance for healing of fractures Vitamin C appears to exert its effect early in the healing process when collagen is formed and we believe that it is the mucopolysaccharide portion of the ground substance which is mainly affected by vitamin C metabolism There is another aspect of healing of fractures in chronic vitamin C deficiency one gets a hyper plasia and hypertrophy of connective tissue cells of whatever origin so much so that you get an occlusion of blood vessels Now with that go certain factors which you have touched on in the nutrition of bone cells I think part of the lack of healing is often due to the occlusion of small arteries That is evidently only in endochondral ossification because in chronic vitamin C deficiency one can get a great increase in ossification around the place of the fracture

Harris I have not studied the healing of fractures in scurvy and vitamin C deficiency My impression has been that there is a tremendous

growth of cells with very scant intercellular substance and under the microscope the cells look like very primitive mesenchymal cells

Follis I think this is in chronic vitamin C deficiency, and as you see it in the human there is constant repair I think that accounts for the increase in tissue that one sees in absolute scurvy in the guinea pig here there is no intracellular material deposited whatsoever However the cells which we call impotent osteoblasts are able to proliferate I do not know by what mysterious means they normally do produce reticulum and perhaps polysaccharide Whereas in chronic scurvy here you have a focus where the scurvy may be absolute and there you have a focus where the scurvy may be just partial and there cells are able to attempt to heal the disease I think that probably accounts for the difference I am not entirely familiar with the vascular changes I would imagine that they may represent secondary responses to trauma and so forth such as are so common in scurvy as a result of fracture

Kodicek I would agree that it would be trauma but microtrauma

Follis Yes we are probably always having microtraumas even when we shake hands

Kodicek But you see this excessive appositional growth in long bones only in chronic scurvy

Follis Some of that is at the insertion of the tendons

Lacroix Dr Harris were your grafting experiments with frozen bone short term or long term experiments?

Harris They were short term and we have only followed them for a matter of a few weeks

Lacroix It might therefore be worth while mentioning here the observations of Axhausen (1952 *Zbl Chir* 77, 435) who claims that frozen grafts are osteogenetic in long term experiments We have per formed similar experiments and we have observed some signs of osteo genesis several months after grafting but we did not publish that because the number of our animals was too small As a matter of fact I think that this particular problem is related to the general problem of osteo genesis by induction and that we would be getting somewhere if the basic problem were better studied In this connection here are a few new facts

In rabbits I have seen that cartilage from a fracture callus is highly and constantly osteogenetic when grafted under the kidney capsule (1953 *Acta chir belg* 52 877) Now the same cartilage keeps its osteogenetic properties when it has been killed by soaking in alcohol for several days Bone appears in the centre of the graft first primary bone then lamellar bone Is it due to the release of some mysterious osteo genetic principle diffusing from the graft or is it the result of a contact action of the grafts on the cells as suggested by Weiss (1950 *Quart Rev Biol* 25, 177)? I do not know But the observation achieves I think its full significance if I add that a rod of epiphyseal cartilage behaves in the same manner that is produces bone whether it is alive or whether it has been soaked in alcohol

In control experiments pieces of rib cartilage are not osteogenetic when they are grafted alive and produce a few bone trabeculae in a

small percentage of cases when grafted dead and observed for a very long time more than one year

I may be mistaken in my interpretation of these facts, but I hope that provided they are confirmed in some animal other than the rabbit they will eventually give us the missing links between bone growth processes bone repair processes and phenomena of heterotopic ossification

Harris Where do you think the bone cells come from?

Lacroix Most of them come from the capsule you can see them streaming from the capsule some come from the kidney parenchyma

Harris So they are connective tissue cells arising from the capsule The rabbit is a notorious animal for producing bone on the slightest provocation The second thing is that the renal system is capable of forming bone on its own

Lacroix I think that the kidney as a grafting bed does not elicit *per se* the osteogenesis observed in the grafts, because the same type of result has been obtained in the anterior chamber of the eye by Urist and McLean (1952 *J Bone Jt Surg* 34A 443) A third set of similar results is now being recorded in my laboratory by a co worker who puts his grafts in the knee joint cavity the cartilage from the fracture callus is embedded in the synovial lining and is transformed into an ossicle the section of which resembles that of a small diaphysis

Harris Have you done any experiments in which the animal was subjected to alcohol treatment?

Lacroix Yes I suppose that you are referring to the experiments of Heinen Dabbs and Mason (1949 *J Bone Jt Surg* 31A 765) who got new bone in the muscles of rabbits by injecting them with alcohol alone I repeated their experiments exactly without ever observing any osteogenesis but I may have failed where someone else succeeded

Let me take this opportunity to stress that there is something peculiar in the whole subject of an alcoholic extract of skeletal tissues being osteogenetic Why is it that let us say half of the authors—including myself—who have tackled the procedure claim that it works whereas the other half deny it? You see now what I am driving at with the experiments I was talking about a few minutes ago instead of working with alcoholic extracts of growth cartilage I prefer to attack the problem from the opposite side that is with experiments deliberately using growth cartilage pretreated with alcohol

Engstrom I do not know anything about all these osteogenic powers but we wanted some sort of bone tissue to use in the study of the relationship between mineral salts and collagen and therefore produced bone by injection of alcohol into rabbit muscles This ectopic bone showed the relationship between apatite crystals and organic matrix discussed before

Lacroix It may be that when you make bone appear where it should not you have put into action some organizing mechanism which is ready to work everywhere or practically everywhere in the organism in the eye the muscle etc but the hypothesis is admittedly far fetched

Meyer I wonder whether Dr Harris has any information about the transformation which takes place in the ground substance in callus

formation Drs P H Maurer and S S Hudack published that in young callus I believe it was 5 day old rabbit callus from experimental fractures, the only polysaccharide which they extracted in relatively high concentration was a hyaluronic acid like substance which presumably was hyaluronic acid In the growing bone when bone formation takes place this material is replaced Now is it certain that there is a different type of cell is it due to a transformation of cells or what is the fate of this material which is replaced obviously by a different type of polysaccharide if we take the polysaccharide purely as an expression of cellular activity?

Harris I cannot answer your question precisely but we think that once one of these osteogenic cells as a very primitive cell has started to differentiate along certain lines it will continue along these lines That is to say it may go on to form cartilage cells and when that tissue later becomes bone, the cartilage which was in the callus has not only been destroyed but also replaced by bone formed by new cells I have no information about the source of hyaluronic acid except that I presume that in the callus these workers were studying they must have taken cells at a very primitive stage and they had not differentiated into bone or cartilage cells and resembled more or less the cells seen in the umbilical cord They were producing a very primitive form of intercellular substance

Follis I think that would be unlikely because as soon as you get appreciable callus to study, you get cartilage like material in it I am not sure that the cartilage in callus has any relationship to ordinary cartilage

Harris Do you think that the intercellular substance in cartilage callus might contain a lot of hyaluronic acid?

Follis I imagine that is the explanation It is an entirely different sort of tissue from classical hyaline cartilage

Nassim Prof Follis we occasionally see these rare cases of myositis ossificans progressiva In those cases where the fibrous tissues all over the body can form bone do they ever go through the intermediate stage of cartilage?

Follis I do not think so

Nassim We have a very distressing case now forming bone all over the place and whenever we section it we see pure bone

Follis Occasionally I have studied it with Dr Howard and we have never seen cartilage

Harris We recently had one case which was thought to be a tumour and according to the pathologist s original report was a chondrosarcoma but I have to admit that the sections did not look particularly cartilaginous Subsequently the true diagnosis became clear as lesions appeared elsewhere in the body

Follis I have been misled by that too on several occasions

Armstrong I should like to ask a question having to do with the hydrodynamics of transcapillary transport in the Haversian system You showed very clearly that each Haversian canal is usually provided with only a single blood vessel Irrespective of whether a given vessel is

an artery or vein, its walls cannot be considered to be permeable since only capillaries are permeable to solutes passing between the plasma and interstitial fluid Even if capillaries are present but are not visualized in your preparations the fact remains that one blood vessel only is present in each canal and a given vessel can be either an artery or vein but not both

Since a single blood vessel cannot serve to provide capillary blood and to collect blood from the same capillaries it is suggested that there is an interosteon transport of blood via capillaries and that the interstitial fluid of neighbouring osteons is in free communication This circumstance would require only that arteries and veins are distributed between adjacent osteons and would also require that ions diffuse over somewhat larger distances than 0 1 mm between adjacent osteons

Harris That is the kind of thing I was hoping to learn when I came here

Armstrong I think that a blood vessel seen in a Haversian canal is passing through the canal on the way to some other place The point is, if a vessel in an osteon is an artery solutes could be delivered from its capillary network but there would be no provision for the entry of solutes from this osteon into the vascular system by way of the same blood vessel

Bélanger Dr Harris you said that the so-called osteoclasts as we know them are not osteoclasts but aggregates of bone tissue which are degenerating In my opinion if we look at the bone spicule from the points of view of its mineral content and its stainability we can see that the mineral content increases progressively to a central point also the stainability with certain dyes particularly phloxin Then the minerals and also the stainability with phloxin decrease towards the tip here will appear a so called osteoclast in the vicinity of which we sometimes find the "brush border" which has been described by Dr Harris If we now look at the osteocytes we see that they are close together at first they get progressively further apart from one another as we reach the central part of the spicule and then again close together at the tip We can now postulate that the spicule appears to become progressively mineralized towards its middle part from then on its mineral content decreases Along with the decrease in the mineral content (which occurs by a biochemical process) some of the ground substance is lost and then the brush border which probably consists of collagen fibres now becomes visible because the ground substance which masked them up to now has disappeared Finally, an irregular mass of organic material with closely related nuclei and no minerals is cast off the so called osteoclast This theory I must say has already been proposed in part by Prof A W Ham (1953 Histology, 2nd ed London Lippincott)

STUDIES ON THE REPAIR OF FRACTURES USING ^{3}P

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IN this paper we report on results obtained by a study of the biochemical mechanisms involved in bone regeneration after fracture Chemical analysis and determination of radio phosphorus fixed by bones were used jointly to follow the different phases of fracture repair

Realization of a definite type of fracture and its adequate fixation are essential conditions for following successfully the evolution of an experimental fracture It is also essential to operate on a rather large number of animals in order to elimin ate individual variations To satisfy these conditions, we per formed a fracture in the middle of a femoral diaphysis, fragments were then fixed by an intramedullary nail, in complete asepsis This way of holding the fracture prevents any displacement and a perfect consolidation is assured within 30 to 40 days No pseudarthrosis has been observed under our experimental conditions One cannot however, exclude a modification of the evolution of the fracture by this treat ment and a study of the influence of the nail on fracture evolution is actually going on Under our experimental condi tions, this method seems to be the most reliable giving constant evolutions which are described in this first paper

Experimental methods and procedures

Adult rats (Wistar strain) weighing 200 g were anaesthet ized with chloral After depilation, the skin was cut and the

fracture was performed by cutting the left femoral diaphysis transversely with a dental burr An intramedullary nail of vitallium (10–15 mm long) was then introduced into both ends of the fractured bone Muscles were sewn together with catgut and the skin was fixed with clips

This mild operation was accomplished under surgical asepsis an initial experiment with less rigorously controlled asepsis was frequently followed by attenuated infections in the zone of fracture

Animals were killed at regular intervals from the third to the fortieth day after fracture Each rat received a total of

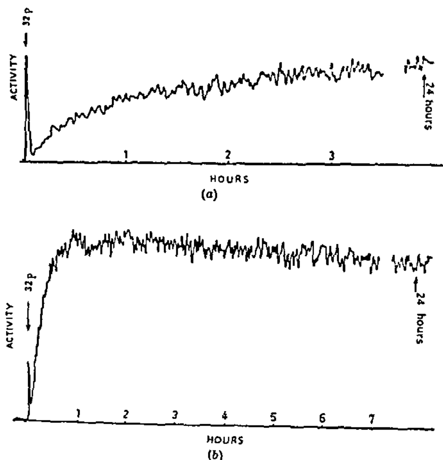


FIG 1 Uptake of ^{32}P by bone *in vivo*

200 μc of ^{32}P (solution of NaH_2PO_4) intraperitoneally 24 hours before death Complete fixation of isotopic phosphorus in the femoral diaphysis was established by measuring bone activity in the living anaesthetized animal maximal activity was reached 1–4 hours after injection (Fig 1), to avoid individual variations, we have chosen the optimum time of 24 hours Repair of the fracture was also controlled by radio grams and histological preparations

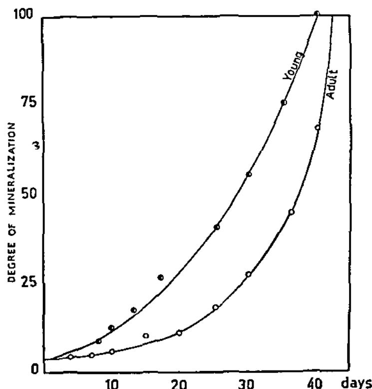


FIG 2 Mineralization of callus

The following bone segments were removed from the sacrificed animal

From the left fractured femur the callus, both terminal parts of the fractured bone a fragment of femoral diaphysis distant from the zone of fracture and near to the inferior epiphysis

From the right femur a fragment taken from the medial part of the diaphysis

The wet weight of these bone fragments was immediately recorded and the phosphorus content determined by the method of Fiske and Subbarow Samples of bone digested with perchloric acid were dried in nickel dishes by an infra-red "epiradiator" To obtain a regular distribution of the

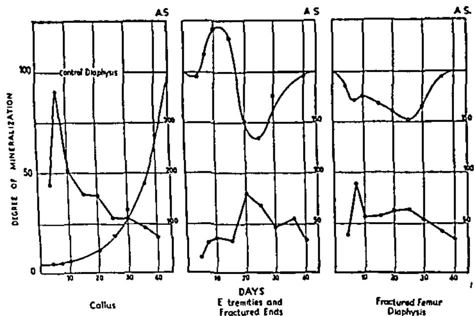


FIG 8 Rate of mineralization and affinity in different segments of bone

ashes the residue was dissolved in a small quantity of 5 per cent acetic acid and again dried Activity of all the samples measured with a Geiger Muller counter, was recorded as the average value of three determinations of at least 2000 counts each By this method we have calculated (1) phosphorus content of the samples, and (2) rate of mineralization of the fractured bone, which was calculated by comparison with the corresponding diaphysis Comparison with the degree of mineralization of normal rat skeletons was impossible because of the great variability of the phosphorus and calcium content

in the rats' bones this was also observed by Roche and Mourgue (1939) and by Dallemagne (1948) Because of the low rate of demineralization which is observed even in the corresponding diaphysis (less than 10 per cent, according to Dallemagne), the comparison between the fractured and the homologous bone is expressed as a ratio which is slightly lower than the real value

To follow phosphorus released during fracture evolution, a balance study of phosphorus and calcium was made on 6 rats during the whole period of fracture repair (40 days) The

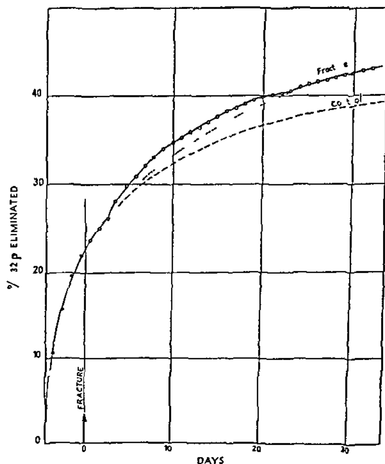


Fig 4 Elimination of ^{32}P in the fractured and control rat

skeleton was labelled with radiophosphorus 5 days before fracture, and faeces and urine were quantitatively collected and analysed daily. This balance was then compared with the normal phosphorus and calcium balance of control rats.*

During the chemical evolution of repair we checked, at every stage, the capacity for phosphorus fixation of the different zones of bone. We defined this fixation by the term "affinity", calculated from the proportion of ^{32}P fixed after injection of $NaH_2{}^{32}PO_4$, 24 hours before removal of the segments.

Results

Experimental results, average values obtained from a series of about 80 rats, are collected in Tables I, II and III and illustrated by Figs. 2 and 3, which indicate more clearly the evolution of fracture repair.

Phosphorus elimination in the fractured and control rats is shown in Fig. 4.

Table I

RATE OF MINERALIZATION OF CALLUS IN YOUNG AND ADULT RATS, figures represent % values of P content of the homologous femoral diaphysis

YOUNG

Days	*Rate of callus mineralization*
8	8.9
10	13.5
13	18.0
17	27.4
25	41.0
30	55.3
35	76.1
40	100.5

ADULT

Days	*Rate of callus mineralization*
4	4.4
7	5.0
10	6.15
15	10.75
20	11.2
25	18.6
30	28.1
36	45.2
40	68.9

* Only one curve for the elimination of phosphorus is given (Fig. 4). The rate of phosphorus elimination was the same for all the animals maintained under the conditions of balance study.

Table II

Rate of mineralization of fractured bone figures represent % values of P content of the homologous femoral diaphysis

Days	*Rate of Mineralization*		
	Callus	*Terminal parts of fracture*	*Fractured femoral diaphysis*
4	4 4	98 0	93 0
7	5 0	109 0	86 0
10	6 15	121 0	88 6
15	10 75	116 5	83 9
20	11 2	76 0	80 0
25	18 6	67 8	75 3
30	28 1	88 2	82 6
36	45 2	92 2	97 2
40	68 9	98 0	—

Table III

^{32}P Activity in different segments of bone figures represent % × 10^{3} of ^{32}P injected

Days	*Rate of callus mineralization*	*^{32}P Activity*			
		Control femoral diaphysis	*Callus*	*Terminal parts of fracture*	*Fractured femoral diaphysis*
4	4 4	23 8	179 2	14 6	39 0
7	5 03	25 1	330 6	30 5	89 1
10	6 15	21 7	204 1	33 4	55 3
15	10 75	21 3	160 7	31 2	58 2
20	11 20	19 7	159 0	79 0	62 8
25	18 6	18 4	113 1	68 3	64 0
30	28 1	18 6	113 2	47 4	53 9
36	45 2	19 5	93 2	55 4	41 6
40	68 9	19 7	73 6	33 9	

Callus evolution

Callus mineralization is accomplished in two phases

(1) during the first 20 days, fixation of phosphorus is small, and it increases very slowly,

(2) from the twentieth to the twenty fifth day onward, rapid mineralization is observed in the fibrocartil aginous tissue formed during the preceding phase the degree of mineralization is about 75 per cent within 10 days

A striking increase of ^{32}P affinity is demonstrated in the callus between the fourth and the eighth day after fracture, subsequently, in relation to the rate of mineralization, a decrease in this affinity has been observed

Evolution of the terminal parts of fractured bone

The whole process is in three phases

(1) during the first 10 days, a significant increase in phos phorus content (20 per cent) is observed The fact that more phosphorus is found than in normal bone indicates that bone condensation occurs in this zone,

(2) from the tenth day onward, the amount of bone salts quickly decreases on the twenty fifth day the degree of demineralization is over 80 per cent,

(3) finally, after the twenty fifth day, the rate of phos phorus fixation increases again in relation to the rate of callus mineralization

Terminal parts of fractured bone show a moderate affinity for ^{3} P, and are highest in the phase of strongest demineraliza tion (twentieth to twenty fifth day)

Evolution of the diaphysis of the fractured bone

Fractured bone shows marked rearrangements during the evolution of callus, as we have observed on analysis of a zone distant from the site of fracture (inferior part of diaphysis)

(1) demineralization of the diaphysis is an immediate conse quence of fracture, the maximum effect is seen towards the twenty fifth day (about 30 per cent),

(2) subsequently, as has been observed in the other zones, mineral fixation begins again, and a normal content of phosphorus is observed at the stage of consolidation of callus.

The specific activity of the diaphysis shows that, in regard to affinity, its behaviour is similar to that of the terminal parts of a fracture: the lower the mineral salt concentration the higher the isotopic phosphorus concentration.

Discussion

Mineralization of callus of the growing and adult animal is accomplished in two phases which are readily identifiable. These two phases, which parallel biochemical processes concerned with normal ossification, seem to be related to different mechanisms.

During the first phase, an equivalent of the initial period of bone formation is evident, i.e., the conversion of the undifferentiated proteid structures to the calcifiable matrix. The arrangement of matrix during this phase is almost without phosphorus fixation. Once mineral binding capacity has appeared, calcium and phosphorus are readily and intensely incorporated, as we have observed in the second phase of fracture repair (twentieth day). During this second period (3 weeks) the phosphorus content increases from an initial value of 5 per cent to the stage of complete mineralization.

This biphasic evolution seems to be present in all the different zones of the fractured bone. A demineralization reaction, in agreement with the first proteid phase, is demonstrated in the diaphysis and in the terminal parts of the fractured bone. The latter segment is, moreover, characterized during the first 10 days by a rather marked condensation, which was also described by Dallemagne (1943) in a study of bone repair in rabbits.

We have demonstrated that a strict relationship exists between the rate of demineralization and the capacity for incorporation of isotopic phosphorus. Analogous conclusions

were drawn by Engfeldt, Engström, and Zetterström (1952), and Lacroix (1953, 1954), who noticed that structures with a low content of mineral salts fix isotopic Ca or P to a high extent.

The determination of this isotopic affinity represents a very sensitive test for demonstrating demineralization processes.* In fact, we have observed that from the first few days following fracture the affinity of the homologous diaphysis increases to a value which is twice the normal (average value 2.3 ± 0.2). This interesting phenomenon, which was also described by Wilkinson and Leblond (1953) seems to be the expression of a general reaction of the whole skeleton to the lesion of a single bone.

Therefore, fracture evolution is not to be considered as a process only concerned with the local mechanisms of repair, but with a more general participation of bone apparatus. This idea is also supported by the result of the study on excretion of radiophosphorus previously fixed by the skeleton. During the first phase characterized by these demineralization processes, evidence is obtained of a remarkable increase in phosphorus excretion in the faeces and urine of rats.

The results obtained by this research have led us to undertake the study of a series of important problems.

(1) Different parts of the fractured bone have shown a marked variation in tricalcium phosphate content, the main problem is to discover the origin of the bone salts we have found in the terminal parts of fracture and the destiny of the salts released during the demineralization phase.

(2) Proteid and mineral phases of the repair of fractures seem to be related to different local mechanisms, the nature of which we do not yet know.

(3) The general participation of the whole skeleton in fracture repair seems to be a stress like reaction: it is

* A striking increase in this affinity (3 times the normal value) follows a degree of demineralization of about 5 per cent (Cartier de Bernard and Lagrange to be published).

important to study the mechanisms (nervous, vascular, hormonal or others) with which it is correlated

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DISCUSSION

Lacroix The larger the experimental animal the more localized are the reactions of its skeleton after a fracture In man between 20 and 50 a fracture of a diaphysis is followed by a definite radiolucency in the metaphysis and in a narrow zone just under the articular cartilage The epiphyseal bone in between is involved afterwards Everything returns to normal if the fracture heals well

At present we are trying to study your subject but in the dog and with microradiographic techniques An aspect which puzzles us is that of some osteons with two concentric zones as shown in an X ray which I have here an outer one poorly calcified and an inner one highly calcified just the reverse of the normal sequence of events What it means we do not know Prof Engstrom tells me that such aspects are sometimes observed here and there but it looks as if they are more numerous in the vicinity of a fracture

Harris I should like to support what Prof Lacroix said from the clinical viewpoint The X ray he shows is a very common finding in fractures occasionally we have had the courage to biopsy the rarefied area and in addition to meaning loss of Ca it means loss of bone substance true osteoporosis Something is happening which inhibits the formation of new bone

Lacroix It may be that the process of osteogenesis is stopped or that there is a leakage of Ca or that there is an exaggerated bone absorption I think it is bone absorption

Harris An easy solution is to regard the body s energy as being concerned with the healing of one fracture rather than with maintaining the normal balance between bone formation and absorption in the rest of the bone

Nassim This is a problem we have been most interested in for a long time There is no doubt that as soon as a fractured limb is immobilized the patient goes into a very negative Ca balance It does not matter whether the whole body is immobilized or not A patient can have a fracture of the tibia and put on a walking plaster and still that patient

will be in a negative Ca balance and putting out about 300 mg of Ca per day I think that is well recognized once you have normal stresses and strains you will not have osteoporosis occurring

Nicolaysen I am rather surprised to hear Dr Nassim s remark in view of the American results where they have people immobilized in casts When Shorr put them in an oscillating bed the retention was back to normal But you observe losses also when they are walking?

Nassim Yes, as long as the limb is immobilized even if they are in a walking plaster they are still putting out 300–400 mg of Ca Also, we have found that when a joint is inflamed for instance a tuberculous knee and it is not immobilized but the person is walking about on a crutch we still find loss of mineral in that limb, not only adjacent to the joint but in the joint above it, in the hip

Dent You are suggesting Nassim that it is chiefly from the part in plaster rather than from the rest of the body?

Nassim Not entirely I am suggesting that for instance in a tuberculous knee which has got say, synovial tubercle and which is not put in plaster where the patient is having only chemotherapy, you will not only have demineralization adjacent to the inflamed joint but you will also get it around the proximal area around the hip He is not weight bearing there

Follis Would you not have more osteoporosis if he were immobilized in plaster?

Nassim Yes

Nordin Surely this is too rapid to be explained by cessation of new growth, although not very much is known about collagen turnover I believe this thing appears very rapidly after the fractures and it seems to me to tie up very nicely with the work of Slack, who denervated and immobilized rat s hind limb and found that there was an increased uptake of labelled glycine into the collagen in the limb This suggests that there is not cessation of new formation but an increased turnover and that the loss is greater than the new formation

Armstrong A fracture is a rather severe trauma Are the effects which you observed and which have been previously noted specifically related to trauma to the skeleton? Would you obtain somewhat similar results with the same degree of trauma to soft tissues? Is this phenomenon a general physiological reaction which is not necessarily specific for the skeleton?

Howard That can be answered very quickly, on overall balance at least because the N loss ceases after about 10 days in an ordinary tibial fracture and the Ca loss continues for about 30–40 days Furthermore it does not happen if you take out a gallbladder or perform other major abdominal surgery Ca does not follow the N balance you get a little loss in Ca but nothing at all like the other

Follis That has to do with immobilization because both with mobilization and with early mobilization everything goes back to normal

Howard It is less marked

Rutishauser We have limited experience with this subject on rhesus monkeys If mobilization is carried out too prematurely decalcification

appears to be more pronounced than is the case when mobilization is carried out cautiously We also injected the stellate ganglion with talc and the results have not been convincing to date

Howard It is interesting that with the tuberculous knee the bone grows faster in a growing child than on the other side That is an old observation which was made in Toronto by Dr Harris s father You can demonstrate it quite readily

Nicolaysen You get that growth in length with aneurysm also Is it all due to circulation?

Harris It must be related to circulation, because every now and then we come across a massive arterio venous communication that has a longer limb on that side

Nicolaysen It happens when you put patients to bed that they sud denly have a very heavy urinary sedimentation with acute kidney attacks and they even become temporarily insane

Blaxter What happens if you produce a fracture in a bone which normally does not bear much weight for example a skull fracture?

Howard You will not get any more negative Ca balance than you would if you operate on a man s stomach

Dallemagne Some years ago we performed a series of experiments of the same kind as those of Drs Cartier and de Bernard We resected sub periosteally a fragment of bone (1 cm) at the middle of the cubitus (or the radius) of the rabbit Some days later we observed a reduction of the Ca and P content in the upper and lower fragment of the resected bone Moreover we regularly observed a decalcification in the diaphysis of the same bone on the opposite side we thought at the time that this phenomenon was depending on a sympathetic circulatory reflex But probably this was not the only mechanism involved indeed this was true when new periosteal bone was formed in the resection focus On the other hand if the periosteum had been destroyed when the resection was performed no new bone appeared and decalcification in the same bone on the opposite side was absent So probably, local and general factors were involved in this process

METABOLIC STUDIES ON VITAMIN D

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When considering the physiological effects of vitamin D and its biochemical mode of action, it is relevant to have information as to the fate of administered vitamin D In fact, this was the usual sequence in the study of mode of action of other vitamins The information about the fate and metabolism of vitamin D is scanty owing to the difficult and laborious assay techniques

It is possible that bile is necessary for the absorption of the vitamin by mammals and that vitamin D is re excreted through the bile (Taylor, Weld and Sykes, 1932, 1935, Greaves and Schmidt, 1933, 1934*a*, 1934*b*, Heymann, 1937*a*, 1938) and probably through the upper third of the small intestine (Heymann, 1937*b*) As to the distribution of vitamin D in the tissues following a dose, only semiquantitative results have been reported (Heymann, 1937*c*) Morgan and Shimotori (1943) concluded that no one organ in particular was concerned with the storage of the vitamin Warkany, Guest and Grabill (1942) appeared to have found in blood almost the entire dose of vitamin D given to patients

The first part of this paper summarizes the results of studies on the distribution and metabolism of vitamin D in rachitic rats following a dose of vitamin D_2 The distribution was determined by biological assay, in collaboration with Dr E M Cruickshank, by quantitative paper chromatography and lately by using ^{14}C labelled ergocalciferol

In the second part, microbiological studies with vitamin D are reported

Methods

The biological assay of tissues of rats was performed by the radiographic method of Bourdillon and co workers (1931) Chemically, vitamin D was estimated by the technique employing reversed phase paper chromatography (Kodicek and Ashby, 1954) Biosynthetically prepared [^{14}C] ergocalciferol, with a specific activity of 3.4 μc/m mole (Kodicek, 1955*a*), and its breakdown products were estimated by the usual isotope techniques combined with reversed phase paper chromatography

The microbiological studies were performed by a procedure previously described (Kodicek and Worden, 1945, Kodicek, 1949)

Vitamin D Balance, Determined Biologically

Groups of weanling rachitic rats were given orally 1 mg ergocalciferol in arachis oil and the antirachitic activity of various tissues and excreta was investigated for 4 days after dosing (Cruickshank, Kodicek and Armitage, 1953) Within the first two days after dosing about 17 per cent of the dose had been excreted in the faeces, whereas no vitamin D was detected in urine (Table I) The small faecal excretion on the last 2 days of the experiment might possibly have been derived from vitamin D re excreted into the gut via the bile as has been observed by Heymann (1937*b*)

Table I

RECOVERY OF ERGOCALCIFEROL (1 MG) GIVEN ORALLY TO GROUPS OF RACHITIC RATS*

	Recovery of dose %							
	24 hr		*48 hr*		*72 hr*		*96 hr*	
Urine	0		—		—		—	
Faeces	14.3		2.7		0.5		0.1	
Liver	3.7	6.0	1.2	3.0	1.0	3.1	1.0	2.4
Intestinal tissue	0.6		0.2		0.2		0.1	
Remainder of body	1.7		1.6		1.9		1.3	

* Biological rat assay Cruickshank Kodicek and Armitage (1953)

The body contained on the first day only about 6 per cent of the dose of which two thirds were in the liver, the body vitamin decreased by half on the second day and remained then unchanged This fall was mainly caused by a decrease in the high initial content of liver The decrease was also noted in other experiments in which vitamin D had been estimated chemically Since there was no comparable rise in other parts of the body, the missing vitamin was either re excreted in the faeces and formed the major part of the excretion on the second day or it was changed into inactive products

The vitamin D content of the intestinal tissue reflected on the first day the amount passing through the alimentary tract, although it was low in proportion to the amount offered However, the vitamin content did not decrease as rapidly as that of faeces, indicating that either vitamin D was removed very slowly from the intestinal tissue or that, in confirmation of Heymann's finding (1937*b*), a certain amount of vitamin D was being re excreted through the intestinal wall

The remainder of the body contained about 28 per cent of the body vitamin and maintained this level throughout the experimental period Muscle, skin and kidneys were the richest tissues (Cruickshank, Kodicek and Armitage, 1954), it should be noted, in view of observations made with labelled vitamin D, that bones had not been analysed separately at this stage of the investigation The other tissues, such as lungs, spleen, brain and adrenals, contributed little to the vitamin content of the body (Table II) The significance of the relatively high amounts found in muscles and skin is at present difficult to explain, the kidneys contained in view of their small size a comparatively large proportion of the body vitamin and, in contrast to liver, evidently retained this amount over a period of two days It is likely that the kidneys act as a haemato urinary barrier, since no vitamin D appears in urine This accumulation in kidneys however, may have another significance, namely, it may be associated with the mineral metabolism of the kidneys, or more specifically with

Table II

RECOVERY OF DOSE OF ERGOCALCIFEROL (1 MG) IN VARIOUS RAT TISSUES*

	Recovery of dose %	
	24 hr	48 hr
Muscle	0 8	0 9
Skin	0 4	0 6
Kidneys	0 5	0 5
Lungs	—	0 1
Spleen	—	0 1
Brain	—	0 03
Adrenals	—	0 01

* Biological rat assay Cruickshank Kodicek and Armitage (1954)

the re absorption of phosphate by kidney tubules (Harrison and Harrison, 1941)

Total Recovery of Dose

Table I shows that 20 4 per cent of the dose of ergocalciferol was accounted for in the body and faeces on the first day For assessing the total recovery, the faecal excretion on the second day (2 7 per cent) has been included, since it may have been derived directly from the dose administered Hence it appears that the total recovery amounted to only 23 per cent The large deficit of 70–80 per cent was evidently caused by destruction or conversion of the vitamin into biologically inactive products It was possible that the destruction might have occurred in the alimentary tract by the action of micro organisms, as has been reported for cholesterol (Turfitt, 1944, Wainfan *et al*, 1952, Stadtman and Cherkes 1952) Succinyl suphathiazole was therefore fed to rats and the recovery of a dose of vitamin D_2 ascertained (Cruickshank Kodicek and Armitage, 1955) It was, however, found that the total recovery in faeces and liver was of the same order, 52 1 and 48 1 per cent respectively, as that of control rats, not receiving the bacteriostatic This may indicate that the destruction of the vitamin does not occur in the gut or that the bacterio static spectrum of succinylsulphathiazole does not include the specific organisms responsible for such an effect

Recovery of Graded Doses of Vitamin D_2

Graded doses of vitamin D_2 (0 25–8 mg) were given to groups of rachitic rats and the vitamin D in livers, kidneys and faeces was determined chemically by the paper chromatographic technique (Kodicek and Ashby, 1954, Kodicek, 1954*a*) The rise in the liver reserves was directly proportional to the dose given (Table III) The amounts constituted about 9–14

Table III

RECOVERY OF DIFFERENT DOSES OF ERGOCALCIFEROL IN TISSUES OF THE RAT*

Group	Dose of Vitamin D_2 mg	Recovery of dose % Faeces 24 hr	Faeces 48 hr	Liver 24 hr	Liver 48 hr	Kidney 24 hr	Kidney 48 hr
1	8	33	2 2	8 0	6 8	0 2	0 2
2	4	20	—	13 5	6 4	0 5	0 2
3	2	17	5 4	11 8	6 8	—	—
4	1	20	5 2	14 0	5 4	—	—
5	0 5	16	3 6	8 8	6 4	—	—
6	0 25	15	3 6	11 6	5 6	—	—

* Paper chromatographic estimation Kodicek (1954*a*)

per cent of the dose, except that at the 8 mg level, which was highly toxic, a relatively smaller rise occurred, comparable to that observed with large doses of vitamin A (Moore, Sharman and Ward 1951), consequently a larger proportion of the dose was found in the faeces (33 per cent) On the second day, the liver vitamin decreased significantly, as observed previously (Cruickshank, Kodicek and Armitage, 1953), to about 40–50 per cent of the amount present on the first day, indicating destruction of the vitamin in liver

In the faeces of rats given doses ranging from 0 25–4 mg, the recovery of vitamin D was, on the first day, 15–20 per cent and, on the second day, 4–5 per cent of the dose, the recovery was thus proportional to the dose given, as was found for liver The ability of the liver to store, after administration of vitamin D, large amounts of the vitamin, far in excess of physiological needs, appears to apply to all fat soluble vitamins (see Kodicek, 1954*b*) The kidneys seem to have a similar property

Studies with ^{14}C-labelled Vitamin D_2

The preparation of crystalline ^{14}C labelled ergocalciferol from [^{14}C] ergosterol, obtained by biosynthesis from yeast (Kodicek, 1955*a*), made it possible to study in more detail the pathway and breakdown of the administered vitamin The tracer experiments were combined with chemical analysis of vitamin D of fractions obtained from various tissues The results confirm remarkably well the data obtained previously by biological and chemical assays Two rats, 10 weeks old, maintained on Steenbock diet No 2965 for 7 weeks, were each given orally 1 mg of 97 per cent pure [^{14}C] ergocalciferol, dissolved in arachis oil, faeces, urine and respired CO_2 were collected for 24 hours The rats were then killed and the pooled tissues and excreta were analysed for the distribution of radioactivity (Table IV)

Table IV
RECOVERY OF ^{14}C ERGOCALCIFEROL (1 MG) GIVEN ORALLY TO RACHITIC RATS

	Recovery %							
	Chemical		*Radioactivity*					
	Vitamin D		*Vitamin D*		*Breakdown Products*		*Total*	
Urine	0	21 0	0	19 7	2 1	61 7	2 1	81 4
Faeces and Intestinal content	21 0		19 7		59 6		79 3	
CO_2	—		—		?		?	
Liver	7 6		5 7	10 6	5 7	9 2	11 4	19 8
Bones	1 4*		(1 4)		2 6		4 0	
Intestines	0 63		0 6		0 9		1 5	
Blood	—		(1 2)		0		1 2	
Kidneys	—		0 2		0		0 2	
Lungs	—		(0 2)		0		0 2	
Brain	—		0		—		0	
Rest of Organs	—		(0 5)		—		0 5	
Muscle and Skin	—		(0 8)		—		0 8	
Total				30 3		70 9		101 2

* Biological rat assay ( ) Chemically not identified

The faeces combined with intestinal content were the only excretory products in which vitamin D was found both

by chemical assay and radioactivity The good agreement between the two analytical techniques is proof of the reliability of the paper chromatographic estimation A large proportion of the radioactive carbon was found in fractions of faeces which did not contain any vitamin D (59 6 per cent), 2 1 per cent of the dose was detected as breakdown products in urine The total recovery of ^{14}C from the excreta was 81 4 per cent, out of which about 20 per cent was present in the form of vitamin D Thus a large proportion of the deficit observed in the balance experiments, studied by biological assay, was found in the excreta, particularly in the faeces It is at present uncertain whether these breakdown products were produced from the administered vitamin directly in the gut, possibly by the action of micro organisms, or were excreted into the intestinal lumen The fact that the liver contained 50 per cent of its radioactivity as breakdown products suggests that some of these substances may have originated in the liver and have been excreted through the bile This possibility would explain the failure of an intestinal bacteriostatic to increase the recovery of vitamin D after dosing The amount of respired CO_2 collected as barium carbonate was so large that it did not allow the detection of ^{14}C derived from the weakly labelled vitamin D.

The body contained about 20 per cent of the total radio activity, half of which was present as vitamin D Again the liver was the organ richest both in vitamin D and breakdown products The bones had 4 per cent of the administered ^{14}C, according to biological assay, made in collaboration with Dr Cruickshank, about half of the radioactivity was present as vitamin D The presence of vitamin D has been detected qualitatively by Russell, Taylor and Wilcox (1934) in bones of chicks given irradiated ergosterol It appears, therefore, that vitamin D is present in structures intimately connected with its physiological effects Whereas the breakdown products in liver were present alike in the unsaponifiable matter and in organic acids, the metabolites in intestinal tissue were entirely in the unsaponifiable matter This may

indicate that the turnover in the liver is different from that of the intestine

The other tissues and blood contained the rest of the dose, most likely in the form of vitamin D

It may be of particular significance that tissues closely connected with the turnover of inorganic phosphate, such as bones, intestines, kidneys and blood contained relatively large amounts of ^{14}C and of vitamin D The distribution by no means paralleled the fat or lipid content of tissues, in agreement with findings of Heymann (1937*c*) Thus, pooled muscle and skin, containing 64 per cent of the body fat, and "rest of organs" (Table IV), with 11 per cent of body fat, had only a small proportion of the radioactive dose Brain contained no detectable radioactivity

Vitamin D in Faeces of Human Subjects

Caution has to be exercised in applying to man conclusions drawn from rat experiments This point is illustrated in the preliminary findings, in collaboration with Drs Cruickshank and Dent, on the recovery in faeces of a dose of vitamin D_2 given to human subjects We have given to 2 normal adults and to 4 patients suffering from osteomalacia, 20 mg ergocalciferol by mouth for 4 days and analysed their faeces, by biological and chemical assay, on the fourth day only about 0 1 per cent of the dose was recovered as biologically active vitamin D (Table V) No difference was found between the

Table V

RECOVERY OF VITAMIN D IN FAECES OF HUMAN SUBJECTS GIVEN 20 MG ERGOCALCIFEROL FOR 4 DAYS

	Recovery % 4th day	
	Chemical assay	*Biological assay*
Normal	—	< 0 0o
Normal	< 3	0 13
Osteomalacia	< 0 6	—
Osteomalacia	—	0 05
Steatorrhea	< 0 6	—
Pancreatic fibrosis	< 0 6	—

normal and diseased subjects The low recovery in faeces is in marked contrast to the relatively large amount of the dose excreted by rats The explanation for the differing behaviour of the two species is at present lacking

Microbiological Studies on Vitamin D

Vitamin D may have an effect on the functioning of intestinal cells in increasing the absorption of calcium, it may exert its influence on bony tissue by facilitating the deposition of bone salts and on phosphate resorbing cells of kidney tubules, but its exact biochemical mode of action is unknown

If it acts at a cellular level, it may be of interest to study isolated cell systems requiring vitamin D Such a condition can be artificially produced in Gram positive bacteria, the growth of which has been inhibited by small amounts (20–80 μ moles) of long chain unsaturated fatty acids, the bacteriostatic activity of the fatty acids increases proportionally to the number of double bonds A reversal of the inhibition can be effected by a number of substances, which are able to change the surface pressure of monolayers of fatty acids The most potent reversing agent has proved to be vitamin D_2 and D_3 (Kodicek, 1948, 1949) Thus vitamin D reverses, proportionally to its concentration (7–12 μ moles/half maximum growth), the bacteriostatic effect on *Lactobacillus casei* of linoleic acid (Kodicek, 1950*a*) Calcium ions have a similar effect, in concentrations of 0 3–0 5 m moles/half maximum growth If the concentration of vitamin D is increased tenfold, the growth of *lactobacilli* is inhibited There is thus a resemblance to the behaviour of mammals towards vitamin D A further similarity is the relation of these responses toward the phosphate concentration in the medium If the concentration of the phosphate is decreased from 26 to 6 5 m moles, the growth-promoting effect of vitamin D or calcium ions, in presence of linoleic acid is enhanced There is, therefore, an apparent optimal Ca/PO_4 ratio but the relationship is only an indirect one The efficiency of the bacteriostatic effect of linoleic acid

increases with increasing concentration of phosphate in the medium Consequently with a given concentration of linoleic acid, an increase in phosphate will result in a greater requirement for vitamin D (or Ca^{++}) to reverse the inhibition, since higher concentrations of linoleic acid or a greater bacteriostatic efficiency will require larger amounts of vitamin D (Kodicek, 1955*b* and *c*)

A number of sterols related to vitamin D have been tested (Kodicek, 1950*b*), ergosterol, 7 dehydrocholesterol, lumisterol$_2$, tachysterol$_2$, and pyrocalciferol$_2$ were inactive in reversing the bacteriostatic effect of linoleic acid Pyrocalciferol and dihydrotachysterol were highly toxic for *L casei* Suprasterol II reversed the inhibition to a certain extent, but dihydrotachysterol (AT10) which has in mammals effects similar to vitamin D, was also fully active for bacteria

Physicochemical Explanation (Barrier Theory)

What is the mechanism of the inhibition of growth and acid production and its reversal in bacteria? It is suggested that these effects can be explained primarily on a physicochemical basis (barrier theory, Kodicek, 1948, 1949, 1950*a*) The active lipoprotein patches of the cell membrane (Davson and Danielli, 1943) are penetrated by the unsaturated fatty acid, after being first adsorbed with its polar group on the surface Because of the double bonding the surface pressure is greatly increased (which would lead eventually to rupture and lysis of the cell), this in turn may distort spatially the protein lattice which appears to form a layer on either side of the (bimolecular) lipoid layer constituting the cell membrane (Danielli, 1954*a*) These portions of the cell membrane are intimately connected with the "facilitated diffusion" in which structural and steric factors are involved, and with the "active transport", requiring free energy such as that derived from enzyme systems (Danielli, 1954*a*, *b* and *c*) The spatial distortion would result in functional changes which may affect the permeation of molecules Such substances as vitamin D may reverse or modify this distortion effect Even if this may be

true for the mode of action in the bacterial cell, it is not known if a similar situation occurs in the mammalian cell

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DISCUSSION

Fanconi I am astonished that in cases of pancreatic fibrosis in steatorrhoea the reabsorption of ergocalciferol was so good because generally we believe that vitamin D is not reabsorbed and in the treatment of these diseases we give vitamin D by injection Did you give water soluble vitamin D?

Kodicek This part of the story could probably be better told by Dr Dent, because he gave the dose, but I shall finish with my part of the story I do not want to claim that it has been absorbed All we can say is that we found negligible amounts in the faeces It could have been destroyed in the intestine It was such a surprise to find such small amounts both in patients and in normal subjects that at the moment I cannot say more

Dent Vitamin D was fed as a tablet of solid calciferol mixed with lactose That is the standard form that we always give our calciferol in We do not give it mixed with calcium phosphate we had a lot of trouble with proprietary preparations which include this compound We did this experiment for the purpose of testing the theory that it is not absorbed in steatorrhoea whether idiopathic or in the form occurring in pancreatic fibrosis I have never believed this although we have been taught it I know of no published evidence, properly controlled that calciferol works better in steatorrhoea when given by injection rather than by mouth In the few trials we have done ourselves we have always noted that it does not work better by injection than by mouth, so we were very glad that we could persuade Dr Kodicek to do this experiment We do not believe this story that it is a fatty substance and that therefore it is not absorbed in steatorrhoea It is a most improbable story as you absorb most of your fats anyhow and you have to assume that 100 per cent of a cholesterol like substance is not absorbed when probably 75 per cent of your other fats are

Kodicek At the moment of course we are arguing in a vacuum because we really do not know if it has been absorbed—or has it been destroyed? It is rather important to realize this

Dent We do know that the ordinary story is wrong the simple medical story that you have steatorrhoea and for that reason you cannot absorb the fat soluble vitamin

Nicolaysen I think you should draw that conclusion from more orthodox fat absorption studies while in steatorrhoea you get 15–20 g of fat in the stool and 80–90 g being absorbed I am very glad you stated this

because it is current in all text books Don't you think your results highly suggestive of post absorptive destruction? You have an exponential curve for the destruction of vitamin D That reminds me of some old experiments done by Leong in dogs with excessive doses of vitamin A He also found an exponential curve at the beginning and then it flattened out

Nordin Surely the fact that you did not recover anything in the stool as vitamin D does not actually prove anything because you have shown in your previous slide that 70 per cent is degraded anyway in the intestine in steatorrhoea it is 100 per cent

Kodicek Yes I wanted to make this clear At the moment we do not know I think the answer can only be found with isotopic tracer studies

Dent We have the clinical evidence that it does not work better by injection at least on such easily measurable things as the plasma levels of Ca, P and alkaline phosphatase

Nordin I thought that the point was that the dosages were quite different, orally and intramuscularly We treat our cases with intramuscular vitamin D which at the level of 100 000 units a month appears to control osteomalacia due to steatorrhoea I understand from the literature that you have got to give something like 100 000 units a day orally to get the same effect in these cases and certainly you can give them 100 000 units a day without getting intoxication If you did that intramuscularly you would get very great intoxication

Dent We never cured an osteomalacia with such a small monthly intramuscular dose but I am sure you can give doses that are comparable to the oral ones and still have no trouble

Nordin That is very interesting because we have got two patients receiving 100 000 units a month intramuscularly, with a high Ca intake and they are recalcifying most beautifully They are up and about and walking and their fractures have healed They both have steatorrhoea they had Milkman s fractures and they had osteoid seams

Dent I would like to see that confirmed with balance data, because that is the only statement I have ever heard that there is a clear cut change when you give this small intramuscular dose

Nassim If I may say so Dr Dent as you have seen balance data we have had an osteomalacia can go spontaneously into remission and as soon as the patient is brought into a metabolic ward and put on a different diet and you do a Ca balance you find he may be in strongly positive balance without having a single extra bit of vitamin D at all and his pseudo fractures heal without any added vitamin D

Nordin I think this is most interesting because we have got two patients who were bed ridden and who remained bed ridden with their Milkman fractures until they had their vitamin D and Ca

Meyer Dr Kodicek in the bacterial experiment have you tried a highly surface active agent like deoxycholic acid?

Kodicek Deoxycholic acid has no effect We have tried about 80 substances Lecithin has a certain effect Certain proteins have some effect also all the substances which form complexes with linoleic acid Desoxycorticosterone and cortisone have no effect

Rogers Just one comment about the accumulation of vitamin D in bones I would like to be quite clear as to what Dr Kodicek is calling bone tissue Does he include the marrow, the epiphyseal plate and so on?

Kodicek In the experiment reported here the term "bones' includes everything also the marrow The next experiment has just been done there we tried as far as possible to take out the bone marrow and we got exactly the same result I did not, of course remove the bone marrow from everywhere but I certainly reduced it

Rogers The majority of that vitamin D or break up product is then, in the bone tissues

Kodicek I would think so

Nicolaysen You might take the marrow fat out

Kodicek Yes but this is not so easy

THE MODE OF ACTION OF VITAMIN D

R Nicolaysen and N Eeg Larsen
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A more comprehensive review was written a short time ago (Nicolaysen and Eeg Larsen, 1953) The interest in the problem has increased, and important advances have been made Here, mostly recent advances will be discussed, although overlapping with the recent article is inevitable

The absorption of calcium

The absorption of calcium is slow in comparison with the absorption of a number of other ions Vitamin D increases the speed of absorption, not only in children and experimental animals but also in adults The effect is clearly seen in osteomalacic patients In adult rats free of vitamin D the absorption remains low in spite of severe Ca deprivation (Malm, Nicolaysen and Skjelkvale, 1955) When vitamin D is next given the absorption reaches the high levels seen in young rats The action of the vitamin on the absorption was observed after only 12 hours in rachitic rats (Lindquist, 1952)

How the vitamin acts on the absorption remains obscure There is some controversy about where in the intestine the vitamin acts Harrison and Harrison (1952) suggest, based on experiments in rats, that the effect of vitamin D in increasing the efficiency of absorption of calcium is observed only under conditions in which the calcium of the intestinal contents is poorly soluble i e, in the lower part of the small intestine Radiocalcium was given by stomach tube and no difference between normal and rachitic rats was seen in the first four hours in which about half of the 10 mg Ca given was absorbed Lindquist's (1952) results (with the aid of the same technique

and a dose of 20 mg Ca) are at variance with Harrison's He observed higher absorption after only one hour in the rats treated with vitamin D Migicovsky and Nielson (1951) gave 1 5 mg labelled Ca to rachitic and vitamin D treated chickens and after only 30 minutes the serum specific activity was 2 7 times higher in the vitamin D treated chickens Nicolaysen (1951) in experiments with isolated loops of the jejunum invariably found a higher speed of absorption in vitamin D treated rats as compared with the negative controls In experiments where the lower part of the small intestine was used for isolated loops, the speed of absorption was found to be slower, however, the rats treated with vitamin D absorbed calcium at a higher speed than the negative controls

The somewhat varying results may be explained as follows Vitamin D influences the rate of absorption of calcium through out the intestine Calcium is absorbed chiefly in the upper part of the small intestine When Ca salts remain in solution in the lower part of the intestine an increased speed of ab sorption can be found in this region too So far, no good evid ence has been produced in favour of the hypothesis that vita min D increases the solubility of calcium salts

The essential problem is how vitamin D promotes the ab sorption of calcium The hypothesis has been put forward that the effect is secondary to the effect of the vitamin on the skeleton The present authors have in vain tried to prove this experimentally

' Phosphates are absorbed quickly and completely in vitamin D *deficient rats given Ca free diets and whatever effect* is observed of vitamin D on phosphate absorption must be considered to be secondary to the increased amounts of Ca remaining in the intestinal contents of rachitic animals

Tubular reabsorption of phosphates

The theory that vitamin D increases the tubular reabsorption of phosphates has in fact been very much in favour since Harrison and Harrison (1941) observed such an effect when

vitamin D was given to rachitic puppies The present authors have discussed this problem recently and no new contributions of decisive value have appeared The reciprocal influence of Ca and inorganic phosphates on the resultant inorganic phos phate and Ca excretion in the urine is well established (Wolf and Ball, 1949, Malm, 1953) We do not deny that vitamin D can influence the tubular reabsorption, however, we maintain that it can only be studied adequately when the amount of Ca absorbed and deposited has been equalized in the animals to be compared Never has a phosphate diuresis been observed in vitamin D deficiency, and never has a depression of phos- phate excretion in the urine following vitamin D administra tion been reported

Table I

THE INFLUENCE OF VITAMIN D ON THE CA AND P METABOLISM IN RATS GIVEN VARIOUS LEVELS OF CA AND P IN THE DIET

SIX 5 WEEK OLD RATS IN EACH GROUP

	Diet 0 25% Ca 0 35% P						*0 96 % Ca 0 35 P*						
	Week	*Δ wt g /day*	*Absorbed mg /day Ca*	*Absorbed mg /day P*	*Urine mg /day Ca*	*Urine mg /day P*	*Δ wt g /day*	*Absorbed mg /day Ca*	*Absorbed mg /day P*	*Urine mg /day Ca*	*Urine mg /day P*	*Week*	
Without vitamin D	1	2 7	14	18	0 3	0 8	1 7	35	9	10	0 2	1	
	2	1 5	15	19	0 4	6 6	1 5	30	5	9	0 3	2	
	3	1 9	19	21	0 5	7 9	0 3	24	5	7	0 4	3	
							1 3	32	8	8	0 4	4	
With vitamin D	4	1 2	23	25	0 9	7 0	0 5	58	17	15	0 2	5	
	5	2 0	24	26	1 2	9 9	1 7	65	18	19	0 3	6	
	Diet 0 48 / Ca 0 35 / P						*Diet 0 49 % Ca 0 57 / P*						
Without vitamin D	1	2 2	19	13	2 1	0 2	1 8	17	25	0 6	5	1	
	2	2 1	17	10	0 8	0 5	2 8	16	30	0 6	13	2	
	3	0 9	16	12	0 7	1 3	1 2	13	27	0 6	15	3	
	4	1 4	19	13	0 7	1 2	0 4	10	23	0 6	14	4	
												Percentage in diet Ca	*P*
With vitamin D	5	1 7	30	24	3 2	2 5	4 1	23	43	1 0	20	0 20	0 41
	6	2 1	34	20	4 0	2 6	2 2	26	44	1 4	20	0 20	0 41
	7						1 2	20	40	0 7	20	0 15	0 36

Diet 85% whole wheat 10% wheat gluten 2% egg albumen 3% brewers yeast 1% NaCl 2% $FeCl_3$

Some experiments performed in this laboratory on rats may be of some use in the discussion The results are given in Table I

The well known effects of varying levels of Ca and P as well as of vitamin D appear clearly In the last series high amounts of phosphates are simply passing through When vitamin D is given, more Ca and P is being absorbed, and even more P is excreted in the urine In fact these observations fit in well with observations in rachitic children given vitamin D

The metabolic pattern of Ca and P

The skeleton in the rachitic state contains less bone salts than normal The problem is how this lesion arises So far conclusive evidence has been produced for rats only A life time study conducted in this laboratory on the Ca metabolism in rats given a diet allowing of optimal rate of growth (Haavaldsen and Nicolaysen, 1956) substantiates earlier work (Nicolaysen and Jansen, 1939, Henry and Kon, 1953) Vitamin D free rats retained practically all the Ca absorbed The urinary Ca remained at a low level in the vitamin D free rats and never exceeded that in rats given the same diet and vitamin D

Defective absorption of Ca is clearly the limiting factor With high Ca/P ratios P may be the limiting factor since its proper absorption is prevented by the excess Ca Urinary Ca may then increase to high values On the other hand, P absorbed over and above what is needed for laying down bone salts, in the proportion Ca/P about 2 2, is excreted in the urine There is no indication at all that variations in tubular re absorption of P are a limiting factor in this respect

'It remains to be established if this holds good also for other species In vitamin D deficiency the net absorption in children and in puppies reaches zero and they succumb to Ca starva tion if not given vitamin D Parenteral feeding of Ca in suf ficient quantitites to maintain bone formation in proportion to the rate of growth may, if successfully conducted, provide the evidence needed

Bone structure

It has been emphasized (Nicolaysen and Eeg Larsen, 1953) that the institution of a vitamin D free state in an animal implies the introduction of three variables at one time Vitamin D, Ca and P deficiency Nicolaysen and Jansen (1939) equalized the supply of Ca and P to the blood stream in rats with and without vitamin D and observed distinct structural abnormalities in the vitamin D free rats Mellanby (1949) reached in principle the same conclusion in rachitic puppies when they received just sufficient vitamin D to promote satisfactory absorption of Ca "Fibrosis" of the bones developed and was cured by higher doses of vitamin D without more Ca being absorbed

Citric acid in bones

Earlier work has been reviewed recently (Nicolaysen and Eeg Larsen, 1953) , and Steenbock and Bellin (1953) and Carlsson and Hollunger (1954) have contributed with extensive studies in rats Steenbock and Bellin reached the conclusion that the amount of citric acid in the bones is related more to the calcium nutrition of the animal than to the rachitic state *per se* Carlsson and Hollunger, on the other hand, found in one series of experiments on rats given a diet with 0 04 per cent Ca that the citric acid concentration in the bones increased by about 70 per cent in the four days following the administration of vitamin D However, they also found that the Ca level in the diet had a considerable influence on the citric acid concentration in the bones In rats given 0 8 per cent Ca in the vitamin D free diet the citric acid was at a concentration 70 per cent higher than in comparable rats given only 0 04 per cent Ca in the diet

The experiments reported by Waasjo and Eeg Larsen (1951, Scand Physiol meeting, not published in detail) may usefully be reproduced (Table II)

The more specific effect of the vitamin on the citric acid concentration in the bones (i e , independent of increase in ash

Table II

THE CITRIC ACID AND ASH CONTENT OF THE FEMUR IN RATS AS INFLUENCED BY VITAMIN D AND THE Ca/P RATIO OF THE DIET

Preparatory period of 15 days on a rachitogenic diet with 0 8% Ca and 0 1% P Vitamin D (10 I U daily) given to the + D group from the 16th day (zero day or week in the table)

Series I Continued on the rachitogenic diet

Days	*Citric acid* %		*Ash of dry wt* %		*Ash / citric acid*	
	− D	*+ D*	*− D*	*+ D*	*− D*	*+ D*
0	0 38	0 38	28	28	74	74
5	0 31	0 50	26	30	84	60
10	0 30	0 68	25	31	83	46
20	0 28	0 66	24	33	86	50

Series II Continued on the same diet but with 0 6% Ca and 0 48% P in the diet

Weeks	*Citric acid* %		*Ash of dry wt* %		*Ash / citric acid*	
	− D	*+ D*	*− D*	*+ D*	*− D*	*+ D*
0	0 38	0 38	30	30	79	79
2	0 31	0 67	53	53	170	79
15	0 54	0 66	63	62	117	94
65	0 54	0 58	62	62	115	107
115	0 53	0 53	62	63	117	118

The figures represent averages of ten

content) appears in the early period following the administration of vitamin D, irrespective of the Ca and P content of the diet In the long term experiment the citric acid concentration in the vitamin D free rats comes close to the concentration found in the rats given vitamin D

✓It appears that we face a dual effect There can be little doubt that the vitamin *per se* influences the citrate accumulation in the bones However, over longer periods of time a defect arising out of vitamin D deficiency is largely compensated for when diets rich in Ca and P are given

On the basis of such a view the somewhat varying conclusions may be reconciled without too much difficulty

Citric acid metabolism

Increase in blood citrate and in urinary citrate following vitamin D administration have been reported lately by Harrison and Harrison (1952) and Bellin and Steenbock (1952) In their latest paper Steenbock and Bellin (1953) present a more detailed study of citrate concentration in various organs following vitamin D administration to vitamin D free rats given various levels of Ca and P in their diet An increase was found in the blood, bone, kidney, heart and small intestine, whereas the level in the liver remained unaffected These observations would indicate a generalized effect of the vitamin on citric acid metabolism and may well mark a new and much more profitable era in the biochemistry of vitamin D

Carlsson and Hollunger (1954) made the following observation when vitamin D was given to rats on a diet with 0 04 per cent Ca and 0 5 per cent P, the citric acid and Ca in the blood increased in parallel, P remaining unaffected When the vitamin was given to rats on a diet with 2 per cent Ca and 0 5 per cent P the citric acid and the inorganic P in the blood increased in parallel, Ca remaining unaffected They interpret these observations as indicative of a primary effect of vitamin D on bone dissolution and feel "forced to assume that citric acid is produced in the bones and that this process is accelerated by vitamin D"

In these experiments Carlsson and Hollunger also reported blood citrate decrease and in some instances Ca and P decrease in the first few hours after the administration of vitamin D

Calcium and inorganic phosphate in the blood

The general view resulting from earlier observations has been that the administration of vitamin D is followed by a fairly rapid increase in serum inorganic P In fact this has frequently been interpreted as indicative of an increased tubular P reabsorption

In rachitic children serum Ca is frequently normal However, when it is reduced in rickets or in osteomalacia (see e g ,

Orr *et al*, 1923, Liu *et al*, 1941) vitamin D administration appears to result in a slow increase only. Lately Carlsson (1952), in work with rats, reported contrary results. Rats given a diet with 0.04 per cent Ca and free of vitamin D had serum Ca values of about 5 mg per cent, when vitamin D was given an increase up to about 8 mg per cent followed in the course of the first few days. The effect was interpreted as due to an increased removal of bone salts. In view of the fact that Nicolaysen (1937) found a distinct decrease of the faecal Ca following vitamin D administration to rachitic rats on Ca "free" diet, a reinvestigation was needed.

In experiments identical with Carlsson's in every respect it was found that the rats without vitamin D on the 0.04 per cent Ca diet absorbed 0.3 mg per day, as compared to 2 mg per day when vitamin D was given. Next, versene (ethylene diamine tetracetate) was added to the diet in increasing amounts to counteract the effect of vitamin D. Although the experiments were not completely decisive it was found that serum Ca always increased in the course of three days following vitamin D administration in spite of very low Ca absorption.

The results presented in Table III, however, would appear to give the evidence needed.

Table III

THE CARLSSON EFFECT OF VITAMIN D ON SERUM CA

Three week old rats for 13 days on Carlsson diet (0.04% Ca and 0.5% P). On the 14th day vitamin D given to group 1 and 0.2% Ca added to the diet of group 2. All figures are averages per day (10 day period)

Group	*Number of rats*	*Treatment*	*Intake*		*Ca mg*		*Serum* Ca mg %*
			Food g	*Ca mg*	*Urine*	*Absorbed*	
1	8	+D 0.04% Ca	6	2.4	0.07	2.0	7.8
2	8	−D 0.23% Ca	6.2	14.0	0.09	6.3	4.9

* Serum Ca on the last day of the balance study in five identically treated rats of each group

The rats given 0.23 per cent Ca in the diet, but no vitamin D, retained about 40 mg more Ca than the vitamin D treated rats on the 0.04 per cent Ca diet, however, their serum Ca

remained distinctly lower [Serum Ca was analysed by the acidimetric method (Nordbö, 1932) which is very precise for small amounts of Ca The standard error of the two sets of values was 0 05]

Thus the amount of bone salts increased but the serum Ca suffered a reduction This might indicate that bone salts of different solubility were deposited in the two groups Whatever the explanation of this observation may prove to be, the fact remains that this "Carlsson" effect lays additional emphasis on the local action of vitamin D *

It is a common experience that vitamin D free rats given high Ca levels in their diet do not develop a low serum Ca

The effect of vitamin D on growth

Most experiments in rats have been performed with diets allowing of stunted growth only The addition of vitamin D has therefore not been followed by any increase in the rate of growth Carlsson (1952) found that his rats on the 0 04 per cent Ca diet increased in weight when they received vitamin D (in comparison with negative controls) He felt this might be referable to the increased serum Ca However, in our experiments reported in Table III, the addition of Ca to the diet had at least the same effect on the rate of growth as the vitamin D administration On the other hand in a life time study on rats (to be reported in full at a later date) in this laboratory on a vitamin D free diet allowing of optimal rate of growth, it was found that the rats receiving vitamin D in addition at the end of the experiment (18 months) weighed about 10 per cent more than the rats given no vitamin D

Summary

The following would seem to sum up our present knowledge of the mode of action of vitamin D

* In the most recent paper (Bauer Carlsson and Lindquist 1955) the view is maintained that vitamin D actively induces bone resorption It is contended that vitamin D does not actively influence accretion of bone salt

The absorption of calcium from the small intestine is increased by vitamin D throughout life The defective accumulation of bone salts is chiefly due to defective absorption Vitamin D also acts in the tissues as evidenced by (1) structural changes in the bones in vitamin D deficiency, (2) citric acid accumulation in the tissues and in the bones following vitamin D administration to vitamin D free organisms, (3) increased serum Ca in vitamin D treated rats in spite of a lower Ca retention than in the vitamin D free rats

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DISCUSSION

Follis How much vitamin D did you give?

Nicolaysen We give them 10 units per day

Follis On this the 14th day (Table III)?

Nicolaysen We generally give them 70 units either in one dose weekly, or twice weekly

Nordin Table III raises another interesting point and that is that the renal threshold for Ca is quite different in the two cases in one case you have got a much higher serum Ca and the urinary Ca is if anything slightly lower In the other case you have got the serum Ca = 4 9 and slightly higher urinary Ca That would tie up with the clinical observa tion that in untreated osteomalacia you have a high threshold for Ca One of the first things you see on giving vitamin D is an apparent change in the renal threshold for Ca You get more Ca appearing in the urine at the same blood level

Nicolaysen We have to be a little careful in interpreting these figures statistically They are on the border line of being statistically significant but you cannot guarantee that in a cage a few μg of Ca are not spilled into the urine

Nordin But even if they were the same you have got such big dif ferences in blood level with no difference in urine That is surely very suggestive

Nicolaysen I would not like to discuss Ca excretion in the kidney In man about 40 g of Ca is circulating through the kidney in the course of 24 hours and 1 per cent error in your estimations will give you all the differences in urinary Ca which can be observed between normal persons

Dent In the rabbit, but not in the human being

Howard 10 g per day, that is the calculated amount of glomerular filtration of Ca per day

Nordin About 97 per cent filtered Ca is reabsorbed

Nicolaysen Yes but the filtrate is only one fifth of the total amount passing through the kidney and therefore if you find 10 1 mg Ca in your blood (or 10 2 mg) and multiply that by five you will get a big difference

Nordin The difference between 4 9 and 7 8 is a tremendous dif ference in blood level The difference in total filtered load per day must be enormous

Dixon I was very interested in this work on vitamin D and citrate that Nicolaysen described It confirms in a vague way what we have always felt that wherever you have Ca transferred across the membrane citric acid in some form may well be concerned in passing it across the membrane whether that is in the intestinal membrane or whether it is in and out of the tubules of the kidney and more and more vitamins and hormones are concerned in potentiating the enzymes It may well be as we have tried to show with parathyroid and citrogenase that in many ways the actions of vitamin D and parathyroid are similar That may be a point in common the potentiating of an enzyme system producing

a local concentration at a particular point where there is transport The data are very vague and it is difficult to prove

Perkins I did some experiments some time ago and worked on citrogenase and aconitase and so on I tried comparing rachitic and normal rats none of which had received excessive doses of vitamin D to determine the aconitase and citrogenase in bones The results we got were somewhat disappointing in that the aconitase was identical in the two groups and in the case of the citrogenase there was possibly a slight effect but nothing definite

VARIATIONS IN SENSITIVITY TO VITAMIN D FROM VITAMIN D RESISTANT RICKETS, VITAMIN D AVITAMINOTIC RICKETS AND HYPERVITAMINOSIS D TO IDIOPATHIC HYPERCALCAEMIA

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To day we are aware of at least 6 different metabolic processes which are, directly or indirectly, influenced by vitamin D

1 It increases the absorption of calcium (primary?) and of phosphate (secondary?) in the intestine

2 It increases the deposition of calcium phosphate in the bones

3 It increases the reabsorption of phosphate in the kidneys

4 It decreases the phosphatase in the blood

5 According to Jonxis, vitamin D furthers the tubular reabsorption of histidine and other amino acids

6 It influences citrate metabolism In rachitic children, the pathologically low blood citrate levels of 1–2 mg per cent show a rise to normal values of 3–4 mg per cent after vitamin D administration In hypervitaminosis D the values climb to 6 mg per cent (hypercitraemia) The favourable influence of citric acid on rickets has long been known It was assumed that citric acid forms a calcium compound in the intestine which is easily absorbed

Many facts suggest that in most if not in all forms of rickets a functional disturbance of the nephron, particularly of the tubule, is involved Sometimes one encounters only one, at other times a complete range of functional disturbances

of the nephron in various intensities and combinations, as in the following illustration of an apparently ordinary vitamin D deficiency rickets (Fig 1)

In this case an ordinary vitamin D deficiency rickets occurred together with aminoaciduria and hyperchloraemic acidosis

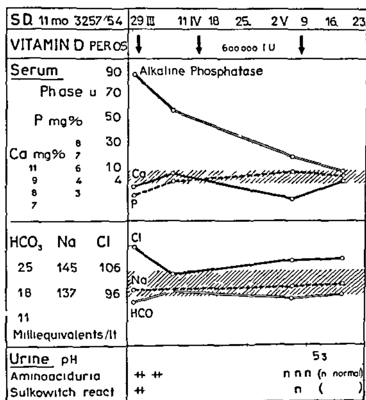


FIG 1 Eleven month old child with vitamin D deficiency rickets aminoaciduria hyperchloraemic acidosis and hyper calciuria All symptoms disappeared on treatment with 3 × 600 000 I U vitamin D given orally

with hypercalciuria Whether we were dealing with a case of Lightwood Albright anacidogenesis cannot be confirmed in retrospect since the original pH of the urine was not deter mined The aminoaciduria also disappeared with the improve ment of the rickets Since we were unable to demonstrate aminoaciduria in the parents we could scarcely consider this

finding as a test for a constitutional rachitic predisposition as suggested by Jonxis

One could consider this case as a transitional form of vitamin D deficiency rickets in a Lightwood Albright syndrome because of the hyperchloraemic acidosis and hypercalciuria On the other hand, the aminoaciduria suggests an insufficiency of the proximal tubule But these alterations of the tubular functions are not the rule in vitamin D deficiency rickets In many cases we did not find any aminoaciduria Probably the ease of alteration of the tubular functions explains why, in our case, a relatively small reduction of vitamin D support was the cause of such a severe rickets, that is, the disposition to rickets consists probably in the disposition to tubular insufficiency

Signs of reduced sensitivity to vitamin D

1 Familial disposition to rickets
2 Familial aminoaciduria (Jonxis)

Signs of increased sensitivity

1 Hypothyroidism
2 Peculiar features of the physiognomy
3 Retarded growth with hypercalcification of the provisional zone of calcification (*Abschlussplatte*)
4 Children with tendency to craniostenosis?

The great range of variation in hypersensitivity to vitamin D on the one hand, and the variation in therapeutic efficacy on the other might be accounted for by the fact that the metabolic pathways which are chiefly affected vary from one individual to another Clinicians have long recognized (1) that there is, on the one hand, a familial disposition to rickets and that on the other, some children never become rachitic, (2) that hypothyroidism and rickets are mutually exclusive, and (3) that rapidly growing children especially, are predisposed to rickets Jonxis recently proposed that amino aciduria may serve as an indication of a predisposition to rickets since he has found aminoaciduria not only in children

having vitamin D deficiency rickets but frequently also in their healthy parents, aminoaciduria would represent a sign of tubular insufficiency

However, it also appears that there are children with a predisposition to hypervitaminosis D Rachitic children toler ate rather large doses of vitamin D and rarely become hyper vitaminotic In contrast, there are infants who are very sensitive to vitamin D and easily develop symptoms of toxic ity—loss of appetite, constipation, dystrophy, hypercal caemia, hypercalciuria and finally severe kidney damage In our early work with de Chastonay we tried to emphasize signs of a predisposition to hypervitaminosis D, as such we recognized hypothyroidism English authors have also noticed peculiar features of the physiognomy of children with idio pathic hypercalcaemia, namely a saddle nose and low placed ears

On the basis of our experience in recent years, I should like to assert further that children with delayed growth, especially those who demonstrate a check line, an *Abschlussplatte* at the epiphyses, that is, an intensive calcification of the cartil aginous ground substance, are hypersensitive to vitamin D It would be important if this assertion were confirmed by further examples, for in these small children compounds con taining vitamin D are often administered with the intention of increasing growth

Furthermore, in a classical case of hypercalciuria we found a closed fontanelle at 10 months and an eminence over the frontal suture In another case—this time a primary idiopathic hypercalcaemia—we encountered a craniostenosis upon which it was necessary to operate I cannot decide whether the premature closure of the sutures occurred as a result of hypercalcaemia or whether it was an indication of a con stitutional predisposition to vitamin D hypersensitivity

For a long time we have known that certain children become hypervitaminotic and therefore hypercalcaemic when given large doses of vitamin D However, not until 1952 did we recognize that there are children who show an idiopathic

hypercalcaemia In collaboration with Schlesinger and co workers from the London school (Fanconi *et al*, 1952) we have described two cases of chronic hypercalcaemia which show the following characteristics

Symptoms of Chronic Hypercalcaemia

1 Dwarfism and mental retardation, convergent strabismus
2 Persistent hypercalcaemia
3 Normophosphataemia or moderate hyperphosphataemia
4 Renal disturbances (moderate albuminuria, persistent azotaemia, lowered urea clearance)
5 Hypercalciuria and moderate hyperphosphaturia
6 Osteosclerosis
7 Alkali reserve slightly lowered
8 Cholesterol slightly elevated
9 Cl and Na normal
10 Asymptomatic congenital malformation of the heart

Table I

CHRONIC HYPERCALCAEMIA WITH OSTEOSCLEROSIS

Date	*Age*	*Length*	*N.P.N*	*Ca*	*P*	*Phosphatase Bodanski units*
19 1 51	2 years	78 cm	70 mg %	13 5 mg %	6 3 mg %	5 9
20 3 55	6½ years	103 5 cm	27 mg %	8 9 mg %	4 8 mg %	18 9

In a follow up of the Zurich case in the spring of 1955 (Table I) it was found that hypercalcaemia and hyperphosphataemia as well as hyperazotaemia and osteosclerosis had disappeared The mental retardation was not improved, however, and, remarkably, the phosphatase measured in Bodanski units remained elevated The systolic murmur was still present In both the Zurich and London case histories there is no mention of normal vitamin D administration, much less of excessive administration

At about the same time, in London, Lightwood described the milder form, "idiopathic hypercalcaemia of infants with failure to thrive" Here, also, we are probably dealing with

children who are extremely sensitive to vitamin D Since then, we in Zurich have seen several similar cases of transitory idiopathic hypercalcaemia which cannot always be distinguished easily from cases of simple D hypervitaminosis (Fig 2)

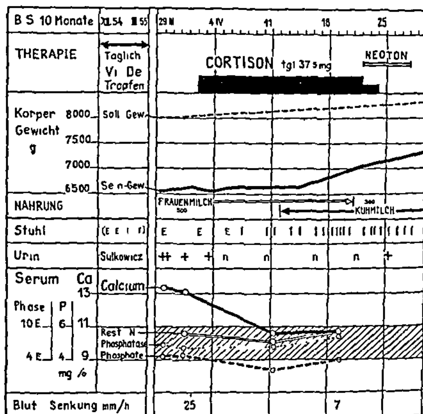


FIG 2 Ten month old infant since birth she received approximately 3 000 000 I U vitamin D orally Following small pox vaccination she presented all the symptoms of idiopathic hypercalciuria and was therefore given a Ca poor diet (human milk) and cortisone after which all the symptoms disappeared but 3 months later the Sulkowitch reaction was again positive and the serum Ca was 11 5 mg per cent

There are, probably *formes frustes* of transitory idiopathic hypercalcaemia also with only moderate serum calcium elevation, but with all the symptoms of the child with failure to thrive, including a positive Sulkowitch reaction

We have also observed the favourable effect of cortisone on hypercalcaemia in a 2 year old boy with severe leukaemia accompanied by widespread bone destruction (Fig 8) Cortisone not only alleviated the severe pain in a short time, but quickly restored the serum calcium value to normal, only 3 weeks later the Sulkowitch reaction which was initially

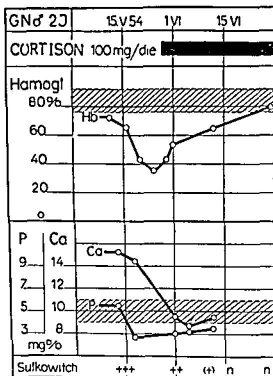


FIG 8 Two year-old boy with hypercalcaemia treated with cortisone See text

strongly positive had become normal In this case the site of action of cortisone was chiefly the bone itself, in that the severe bone destruction was checked through inhibition of the proliferation of paraleucoblastic cells Anderson and Dent also conclude that cortisone improves hypercalcaemia as, for example, in sarcoidosis, through inhibition of calcium re-absorption in the intestine They consider cortisone, therefore, as a vitamin D antagonist

We now recognize a series of pathological conditions in childhood which are differentiated by their completely different reactions to vitamin D (Fig 4) At one extreme lies vitamin D resistant rickets, at the other, chronic hypercalcaemia It would be false to conclude, however, that the spectrum of vitamin D sensitivity is continuous, that is, that all transitional stages between the various clinical syndromes exist We must rather conclude that the pathogeneses of the

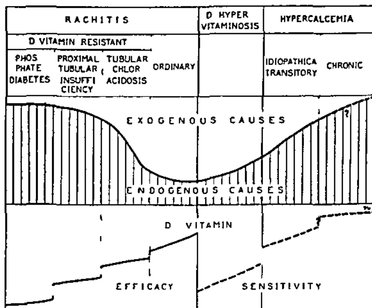


FIG 4 Spectrum of varying reactions to vitamin D

various forms of vitamin D resistant rickets as well as of ordinary rickets are also varied Likewise, various mechanisms might play a rôle in the occurrence of transitory hypercalcaemia and in the still rarer form of chronic hypercalcaemia We have seen that vitamin D works in at least six different places in metabolism It is possible even probable, that the sensitivity of these various end organs differs from one individual to another

It is further conceivable that there is an *affinité majeure* for vitamin D which manifests itself in a prompt elevation

Table II

VITAMIN D RESISTANT RENAL RICKETS

Name	Part of the nephron involved	SERUM						URINE					
		Ca	P	P'tase	N P N	Cl	CO_2	Ca	P	Conc Power	Gluc	Amino acids	pH
Hyperphosphataemic renal rickets	Glomerulum	L	H	H	H	N	L	N	L	I	—	—	N
Renal hyperchloraemic acidosis (Lightwood Albright)	Distal tubule	N	NL	H	N	H	L	H	N	L	—	—	H
Debré de Toni Fanconi syndrome with and without cystinosis	Proximal tubule	N	L	N or H	N or H	N	L	L	N or H	L	+	+	H
Phosphate diabetes (Primary vitamin D refractory rickets)	Part of the proximal tubule?	NL	L	H	N	N	N	L	N or H	N	—	—	N

N = Normal L = Low H = High

of the serum phosphate level and that there are several *affinités mineures* For instance, in vitamin D resistant rickets the *affinité majeure* is eliminated and therefore hypophos phataemia persists, the vitamin can only work through the *affinités mineures*, whereby much larger doses are necessary

We recognize at least four forms of vitamin D resistant rickets of renal aetiology (Table II)

1 The glomerular hyperphosphataemic form, which soon leads to secondary hyperparathyroidism In childhood, especially, one finds osteomalacia secondary to the acidosis in addition to fibro osteoclasia Vitamin D seems to be contra indicated in these cases

2 The forms of tubular aetiology are

(a) Insufficiency of the proximal tubule, which is character ized by aminoaciduria, relative phosphaturia and glycosuria Often, but not always, this proximal tubular insufficiency is associated with cystinosis Also, a disturbance of acid pro duction is the rule

(b) Primary vitamin D resistant rickets, or phosphate diabetes, in which only the tubular retention of phosphates is disturbed The characteristic feature of both forms, (a) and (b), is the low blood phosphorus which is very difficult to raise

(c) Rickets in Lightwood Albright's hyperchloraemic renal tubular acidosis Here the serum phosphate is normal and, in contrast to forms (a) and (b), the Sulkowitch reaction is strongly positive In the progressive form nephrocalcinosis develops

One can also attempt to define the various forms of renal rickets on the basis of disturbed partial functions of the nephron From a scientific point of view this would be more correct, for in a concrete case the intensity of disturbance of the single partial function varies, and combinations other than those shown in Table III may be present

In a case of cystinosis, we succeeded not only in improving the general condition by vitamin D, a Cl poor diet and alkali therapy, but the tubular symptoms also became normal with

Table III

FORMS OF RENAL RACHITIS SECONDARY TO DISTURBANCE OF PARTIAL FUNCTIONS

Disease	*Glomerulum*	*Proximal tubule*			*Distal tubule*	
		phosphate	*amino acids*	*dextrose*	*acido genesis*	NH_3—*production*
Glomerular hyperphosphataemic rachitis	+	n	n	n	n	n
Debré de Toni Fanconi syndrome with and without cystinosis	n later +	+	+	+	+	?
Phosphate diabetes	n	+	n	n	n	n
Lightwood—Albright tubular acidosis with and without nephrocalcinosis	n later also +	?	n	n	+	n or +

n = normal + = disturbed

the exception of the ability to concentrate In contrast, we did not improve the cystinosis or the hyperazotaemia, the bone marrow, before and after treatment, was rich in cystine crystals With the intention of combating the continuing severe anorexia, constipation, and poor weight gain we tried treatment with cortisone in analogy to idiopathic hypercalcaemia which causes the same clinical symptoms This reasoning was wrong because cortisone works as an *antagonist to vitamin D and the vitamin D injections* had influenced the proximal tubular insufficiency and the bone changes very favourably in this case Accordingly, the condition in no way improved and the child died suddenly Autopsy revealed a severe cystinosis

Another child was considered for years as a case of atypical phosphate diabetes without other kidney symptoms until X ray examinations disclosed a severe nephrocalcinosis Exact analysis revealed that we were dealing with a Light wood Albright syndrome of anacidogenesis with hypercal ciuria The fact that vitamin D quickly raised the serum phosphate level to 5 5 mg per cent and that the Sulkowitch reaction was positive suggested that this was not a case of phosphate diabetes With a Cl poor diet alkali therapy and vitamin D the child improved greatly (Table IV)

In proximal as well as in distal tubular insufficiencies the combination of alkali therapy with 1–2 injections of 600,000 I U vitamin D per month has been sufficient to improve considerably the rachitic bone changes In contrast, in phosphate diabetes much larger doses of vitamin D must be administered e g , 1–2 million I U per week Here only the ability to maintain a normal value of serum phosphate is lost

Symptoms of Renal Phosphate Diabetes
(Primary Vitamin D resistant rickets)

1 Familial, usually with dominant genetic pattern
2 Chronic absolute vitamin D resistant hypophosphataemia
3 Phosphatase elevated before vitamin D therapy
4 Normo or hyperphosphaturia

Table IV

NEPHROCALCINOSIS

B W 5 years J No 0331/55, Case No

Diet		*Normal*		*Low NaCl*	*Low NaCl + 6g Na citrate*
Date		23 3 55	24 4 55	19 4 55	29 4 55
N P N	mg %	29	26	—	26
Ca	mg %	11 7	9 0	10 7	10 2
P	mg %	4 3	5 2	4 8	4 5
P tase	mg %	9 6	—	—	6 7
Na	m equiv /l	146	140	140	144
K	m-equiv /l	6 0	4 5	3 0	4 7
Cl	m equiv /l	112	110	102	103
CO_2	m equiv /l	13	16		27

Phosphate clearance high The Ellsworth Howard phosphaturia test shows only minimal increase in phosphate excretion

5 Pronounced hypocalciuria
6 Appearance after the first year
7 Retarded growth, sturdy body configuration

A persistent hypophosphataemia is remarkably combined with a normo or indeed hyperphosphaturia, so that the phosphate clearance is very high This was the basis for my having spoken of "phosphate diabetes" Since our first patient which I described with Girardet in 1952, we have studied 11 additional cases In 3 families we found a dominant hereditary pattern where the mother and one or two children were affected

To obtain a rickets healing effect much higher doses are necessary Yet vitamin D intoxication is also possible in phosphate diabetes This was the case in a 4½ year old girl

whom we observed It is, therefore, urgent in the treatment of vitamin D resistant rickets to carry out the Sulkowitch test regularly in order to interrupt the vitamin administration at the proper time

The name "renal phosphate diabetes" suggests that the basic disturbance rests in the inability of the renal tubules to respond to vitamin D and possibly also to parathormone In fact, we found in our first case that Ellsworth Howard's phosphaturia test is practically negative as in pseudohypo parathyroidism While, however, too little phosphate is excreted in pseudohypoparathyroidism so that a hyperphos phataemia and a secondary hypocalcaemia occur, in phosphate diabetes the kidneys allow much too much phosphate to pass through, so that hypophosphataemia results In contrast to our viewpoint, Zetterstrom conjectures that the disturbance in primary vitamin D resistant rickets lies in the absorption of Ca phosphate in the intestine This would explain also the greatly decreased calcium excretion in the urine, but the restricted absorption of Ca in the gut could also be a con sequence of decreased calcification of the bones caused by the constant hypophosphataemia

The familial incidence of phosphate diabetes indicates that we are dealing with a hereditary disease, but the dis turbance often manifests itself for the first time some years after birth We have at our disposal an observation of a case of phosphate diabetes which was first noted after puberty and which reacts very well on 1 2 million I U vitamin D per week (Fig 5)

The internist would speak in this case of adult osteomalacia (Wernly, 1952) I postulate that it is apparently, a phosphate diabetes which first became manifest in puberty I venture to suggest this even more strongly since the mother of our first studied case of phosphate diabetes had rickets as a small child but developed severe osteomalacia first in puberty and again during pregnancy At times when calcium and phos phate metabolism is not increased, phosphate diabetes can occur without manifest symptoms



Fig 5 (a) 17 9 1954



Fig 5 (b) 7 5 1955

Acquired hypophosphataemic osteomalacia

	13 vii 54	17 ix 54	23 xii 54	7 v 55
Ca mg %	9 3	11 4	10 5	8 9
P mg %	1 3	1 4	3 1	2 6
Phosphatase Bodanski units	5 9	9 3	16 2	12 2
N P N mg %		18	26	35

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DISCUSSION

Kodicek There are two points which I would like to mention in connection with osteomalacia

The first arose from discussions with Dr Dent in which we wondered what happens to the large doses of vitamin D given to patients with osteomalacia Where does the vitamin go? It was in the beginning of our investigations on the fate of administered vitamin D and we did not know then that the vitamin is not excreted in urine On chromatographing fractions of ether extracts of urines of subjects dosed with 20 mg vitamin D no vitamin D or related sterols were found But instead, in urines of patients suffering from vitamin D refractory osteomalacia whether dosed with vitamin D or not several abnormal substances were observed in the chromatograms two of which appear to be Δ^4-3 ketosteroids not identical with cortisone cortisol corticosterone or its 11 deoxy 11 dehydro 11 deoxy 17α hydroxy derivatives testosterone or progesterone So far urines of 7 patients were investigated and compared with those of 7 normal subjects in every case the abnormal spots were noticed in the urine of the patients

The following procedure was used urines collected for 24 hours were extracted repeatedly with ethylether the lipid extracts were saponified

and fractionated by passing through a MgO Kieselguhr column using 0 75 per cent acetone in light petroleum as the solvent The unabsorbed weakly polar fraction was collected and put on paper the absorbed polar fraction was eluted with acetone and ethanol and also chromatographed Fig 1 shows a representative example of the

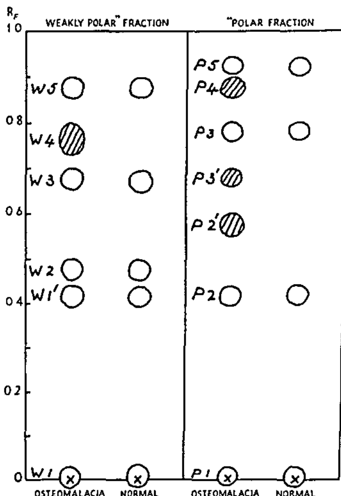


FIG 1 (Kodicek) Reversed phase paper chromatograms of fractions of ether extracts of urines of normal subjects and patients suffering from osteomalacia Weakly polar fraction not adsorbed on MgO Kieselguhr column Polar fraction adsorbed on MgO Kieselguhr column

Both fractions run on paraffin impregnated paper with methanol n propanol water (80 15 5)

chromatograms The paper chromatography was performed on paraffin impregnated filter paper, Whatman No 2, the developing solvent was methanol n propanol water (80 15 5) The paper was sprayed with $SbCl_3$ In the 'weakly polar' fraction the abnormal spot W4 and in the polar fraction the abnormal spots P2 P3' and P4 were visualized Spots W4 and P4 gave a brown colour on $SbCl_3$ spray, were fluorescent in longer wave ultraviolet and dark absorbent in short wave ultraviolet light (256 mμ) They gave a strong p phenylene diamine reaction and a yellow fluorescence after NaOH spray, all of which are characteristic of α β unsaturated ketosteroids Spots W4 and P4 had an R_F of 0 94 and 0 16 respectively, in Bush's system using alumina impregnated paper with benzene methanol water (10 5 5) as developing solvents

These spots thus give all the reactions of Δ^4–3 ketosteroids they may be identical with some of the unknown adrenocortical steroids described by Gray and Lunnon (1953 *Mem Soc Endocrin* **2** 64) It is of course tempting to speculate in view of the clinical interaction of cortisone and vitamin D that there is some disturbance of adreno cortical function at any rate in the osteomalacic patients examined

This brings me to my second point Dent suggested that the beneficial effect of cortisone in patients with hypersensitivity to vitamin D could be considered as an antimetabolite effect But I am afraid that the structure of vitamin D is so different from that of cortisone that one could not speak of cortisone as being an antimetabolite in the conventional sense To test experimentally the possibility of an interaction we have in collaboration with Dr Cruickshank produced by daily doses of 1 mg vitamin D a hypervitaminosis in young growing rats and treated one group with 1 mg cortisone acetate intramuscularly daily Table I shows that cortisone treatment did not ameliorate the

Table I (KODICEK)

EFFECT OF CORTISONE ON TOXICITY OF VITAMIN D_2 IN RATS
(Cruickshank and Kodicek unpublished)

	Groups (8 rats each)		
	Hypervitaminotic	*Hypervitaminotic + cortisone*	*Cortisone controls*
Treatment	1 mg vitamin D_2 daily	1 mg vitamin D_2 1 mg COAC* daily	1 mg COAC* daily
Number of animals died	1	6	0
Calcifications in organs	++	++	0
Urinary P mg /day	3 0	2 7	0 14
Ash content of bones (fat free)	41%	43%	48%

COAC = cortisone acetate

weight loss clinical appearance histological lesions or the increased urinary phosphate excretion of the hypervitaminotic rats It is quite true that rats may differ from man in their behaviour towards cortisone also that the ratio of doses of vitamin D to cortisone acetate was 1 1 while in patients greater quantities of cortisone are given We were not able to increase the dose of cortisone because of toxic effects in the rat However we believe that the results indicate that there is not a *direct* "antimetabolite ' effect of cortisone towards vitamin D

Nassim One point was raised on the treatment of these people with vitamin D resistant rickets We found similarly that it is very difficult to get growth because the amount of vitamin D you have to give leads to toxaemic symptoms before you have managed to raise the serum P to childhood growing levels and so they do not grow In metabolic balance studies upon six patients with resistant rickets we demonstrated (Saville P D Nassim J R Stevenson F H Mulligan Lily, and Casey, Margaret (1956) *Clin Sci* , in press) that AT10 has a Ca and P retaining effect in active cases It is concluded that its action is qualitatively and quantitatively similar to that of calciferol The addition of large doses of sodium phosphate to the diet increased the Ca and P retaining effect of AT10 in all cases and lowered serum Ca levels In one case added dietary P alone appeared to improve intestinal absorption of Ca

Black I would like to go back to the hypercalcaemia that Prof Fanconi mentioned Recently Hallman and Ylppo (Hallman N and Ylppo A (1954) *Acta Paediat* 43 Suppl 100 p 382) have thrown some light on it from a clinical point of view in that they have shown that this hypercalcaemia syndrome has developed in a number of children who were originally premature and who developed interstitial plasma cell pneumonia and were treated with variable doses of vitamin D They all developed exactly the same symptoms that Prof Fanconi has described We have also been very interested in the question of mental defect Some of Hallman s cases were mentally retarded up to many months after the original illness and after the Ca had returned to normal That is something we have observed in similar cases without any pneumonia in this country We followed some of the children up to the age of 4 or 5 they were still mentally defective (Schlesinger B E Butler N R and Black J A (1956) in press) and we have no adequate explanation for it

Dent It looks as if there are going to be many more syndromes in which sensitivity to vitamin D plays a part This is a completely different disease I myself cannot see any metabolic difference between vitamin D intoxication and the syndrome which is described as the hypercalcaemia syndrome

Nassim In regard to the infants who get better quickly is it true that practically all of those described have been artificially fed?

Dent Most but not all The one you showed in Fig 2 was breast fed wasn t he Prof Fanconi?

Fanconi No he received human milk only in the hospital and was not breast fed before We give breast food because mother s milk has a

low Ca All our children were treated at home with cow s milk, whereas the Helsinki children were breast fed But these received very high doses of vitamin D for the prevention of rickets and I believe that most premature infants are hypersensitive to vitamin D Perhaps you see hypercalcaemia much more in England than in America because the milk in England contains 1000 per quart, and in America only 400

Nassim That is what I was thinking might be a possible cause

Dent Presumably when they are weaned they develop hypercalcaemia

Black We have had some cases in which the symptoms appeared to develop while the children were still breast fed

Blaxter It is interesting that in young suckling calves we have come across a very small number of cases which I think are quite comparable They habitually showed a failure to thrive and grow, the serum Ca was elevated to 15 or 16 mg /100 ml At postmortem there was some high degree of metastatic calcification and the kidneys were grossly abnormal We have done no metabolic work on them but I think the condition is probably comparable These calves were receiving their dams' milk and were out in the sunshine so presumably if it is a vitamin D effect there must be individual sensitivity in the calf also

Follis I think Dr Kodicek's experiments ought to be done on other animals for we know the rat responds in a unique way to cortisone, unlike any other animal I know

Kodicek Which animal would you suggest?

Follis As a matter of fact cortisone has very little effect on any animal except the rat the opposite to what you would expect

Kodicek The point is that we were after a direct antagonism between vitamin D and cortisone If there is a direct antagonism in terms of an antimetabolite action we should have seen it

PRESENT KNOWLEDGE OF PARATHYROID FUNCTION, WITH ESPECIAL EMPHASIS UPON ITS LIMITATIONS

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As I understand it, conferences of this kind have been designed to bring together individuals of widely diverse scientific disciplines, but each participant is interested in that compartment of biological economy to be under discussion At such a gathering the contribution of a clinician cannot be so precise or mathematically documented as those of his confrères in the spheres of anatomy, chemistry or biophysics Nevertheless, before a physical or chemical concept can be accepted as applicable to a problem in the living man, it must be found not to run counter to observations on the natural history of disease states and on the anatomical and chemical observations which accompany natural phenomena From his observation post the clinician has sometimes been able to offer useful clues which have helped in the final understanding of a physiological process It is in this spirit that the following remarks will be made, based for the most part on inferences derived from the simplest sorts of observation, deduction and experiment

The historical evolution of our current knowledge of para thyroid function may be said to rest on three major steps

(1) The tetanic spasms and convulsions which sometimes follow thyroidectomy were observed to occur only when the parathyroid glands had also been removed (Gley, 1897)

(2) MacCallum and Voegtlin (1909) discovered that the serum calcium falls after parathyroidectomy, and that the

symptoms of parathyroid insufficiency can be prevented and alleviated by administration of calcium salts, as well as by injection of crude extracts of parathyroid glands

(3) Collip (1926) prepared a sterile, reasonably stable, physiologically active extract of the parathyroid glands which can be used for experimental purposes

Our current knowledge of what rôle the hormone (or hormones) of the parathyroid plays in biological processes stems mainly from three sources The first of these is *parathyroidectomy*, experimental or inadvertently produced by surgery in man The second is spontaneous hyperparathyroidism, either adenoma or primary hyperplasia, the two being indistinguishable clinically In accepting data derived from spontaneous hyperparathyroidism for use in defining normal parathyroid function, one must of course make the premise that the parathyroid adenoma secretes merely an excess of the hormone(s) produced by the normal glands It seems likely that this is so since the biochemical changes in hyperparathyroidism are strikingly the converse of those found after parathyroidectomy The third source of information stems from experiments using parathyroid extract Though this substance is extremely crude and highly impure, there is good reason to believe that it contains one or more principles which have the same physiological activities as does the secretion of the normal parathyroid gland, for, in man, large doses produce the same symptoms and the chemical changes in the serum as those found in spontaneous hyperparathyroidism (Johnson and Wilder 1931), and in the experimental animal there also results osteitis fibrosa indistinguishable from that which accompanies *parathyroid adenomata* Furthermore, the biochemical changes of hypoparathyroidism can be restored to normalcy by appropriate doses of parathyroid extract It seems justifiable, therefore, to deduce that parathyroid extract contains the active principle of the normal parathyroid gland

From the sources mentioned above it is concluded that parathyroid hormone operates in three biological areas

I Phosphorus Metabolism

Before an audience of this kind there is no need to discuss the transport mechanisms of calcium and phosphate in the body fluids (Howard, 1953) For purposes of simplicity serum protein will hereinafter be considered to be normal, hence hyper or hypocalcaemia will be presumed to mean higher or lower than normal concentration of calcium in the interstitial fluid Serum and urinary phosphorus will mean inorganic phosphate of these fluids and the phosphorus will be considered to be wholly diffusible

The first functional alteration to be noted after administration of parathyroid extract is phosphorus diuresis (Albright and Ellsworth, 1929) In the normal man or animal this phosphorus diuresis may be slight and largely accounted for by increased glomerular filtration rate (Jahan and Pitts, 1948, Handler, Cohn and de Maria, 1951, Milne, 1951), but in the hypoparathyroid subject the degree of increased phosphaturia is far greater than can be accredited to this source (Ellsworth and Howard, 1934, Handler, Cohn and de Maria, 1951) One gains the distinct impression that tubular phosphorus resorption is sharply influenced by normal quantities of circulating parathyroid hormone, and that quantities of hormone above the normal are relatively much less effective in this regard (Howard, 1953)

It seems quite certain that the hormone is one of the factors in determining the concentration of phosphorus in the serum In hyperparathyroidism the serum phosphorus is lower than would be the case under otherwise similar circumstances, in hypoparathyroidism serum phosphorus is higher

II Action on Calcium Metabolism

Hyperparathyroidism is associated with hypercalcaemia (as previously defined) or at least with a serum calcium higher than it would otherwise be But the most dramatic evidence stems from the prompt fall in serum calcium which follows

parathyroidectomy Hypercalciuria usually accompanies hyperparathyroidism and hypocalciuria is seen in hypoparathyroidism, but whether this is only a reflection of corresponding serum changes or a direct effect upon renal excretory mechanisms seems doubtful at the moment The former possibility appears to us the more likely

III Action on Bone

It was at one time suspected that the effects of the hormone on both serum calcium and on bone were produced indirectly through the changes in phosphorus metabolism These suspicions were dispelled when it was demonstrated that both hypercalcaemia and osteitis fibrosa resulted from administration of the extract even when urinary excretion was prevented and the serum phosphorus was consequently rising steadily (Ellsworth and Futcher, 1935, Ingalls, Donaldson and Albright, 1943) But most convincing of a direct parathyroid action were the experiments of Barnicot (1948), confirmed by Chang (1951) Transplants of parathyroid tissue beneath the periosteum resulted in local areas of osteitis fibrosa

Stimulus to Parathyroid Activity and the Rôle of the Parathyroid in Calcium Homeostasis

It appears that the level of serum calcium is an important governor of the rate of parathyroid hormone secretion Experimental rickets (normocalcaemic) produced in the usual fashion by high calcium and low phosphorus diets is not accompanied by parathyroid hyperplasia, whereas hypocalcaemic rickets resulting from low calcium diets does cause parathyroid hyperplasia (Ham *et al*, 1940) Enlargement of a gland does not of course prove increased secretory activity, but Patt and Luckhardt (1942) provided direct evidence by perfusing isolated thyroid parathyroid preparations and found that low calcium perfusions produced perfusates with greater parathyroid activity

Indirect evidence that the serum calcium level controls parathyroid output in man was afforded by experimental hypercalcaemia induced by injection of calcium salts Serum phosphorus rises as a result of this procedure (Baylor *et al*, 1950), yet in the normal individual urinary phosphorus excretion falls (Howard, Hopkins and Connor, 1953) An increased serum phosphorus together with reduced urinary phosphorus is characteristic of hypoparathyroidism, and the tentative conclusion was that hypercalcaemia reduces para thyroid secretion This conclusion was fortified by finding that, when similar injections of calcium salts were given to patients harbouring parathyroid adenomas and to hypopara thyroid subjects, the serum phosphorus also rose but in these cases phosphorus *diuresis* occurred Nordin and Fraser (1954) provided corroboration for these deductions by infusing cal cium and parathyroid extract coincidently into normal sub jects and finding that under these conditions phosphorus diuresis accompanied the serum phosphorus rise

There are those who believe elevation of serum phosphorus is also a stimulus to parathyroid function (Crawford *et al*, 1950) Our present view is that this mechanism need not be postulated to explain currently available facts Though many persons dying of chronic renal insufficiency and hyperphos phataemia are found at postmortem to have enlarged para thyroids (Pappenheimer and Wilens, 1935), we have not yet encountered primary renal insufficiency accompanied by hypercalcaemia, a situation which ought to have been met if hyperphosphataemia were a potent parathyroid stimulant for hypercalcaemia is seen with parathyroid adenomata even in the face of renal insufficiency and hyperphosphataemia (Elsom, Wood and Ravdin, 1936, Baker and Howard, 1936)

The evidence, therefore is that hypercalcaemia reduces or turns off parathyroid secretion, and that hypocalcaemia is a stimulus to parathyroid activity, ablation of these glands results in hypocalcaemia and their overactivity in hyper calcaemia The deduction naturally follows that the para thyroids play an important rôle in calcium homeostasis which

McLean and Hastings (1935) have described as "one of Nature's physiologic constants"

Limitations of our Knowledge Regarding Parathyroid Function

Some thoughts will be offered later as to where and how the parathyroid hormone may act in carrying out its calciostatic function, but at the moment it is fair to say that we do not know the chemical structure of the active principle of parathyroid extract, we do not know the precise site or sites of its action, we do not know whether there may be one or multiple active components, and we do not know whether the material as secreted must be changed in some way by the body into an active form No enzyme system has, to our knowledge, ever been shown to respond to the extract *in vitro*, a field well worthy of study and one in which we ourselves have been making, so far, futile attacks Chemical dissection of parathyroid extract has been attempted The efforts of Handler, Cohn and Dratz (1953) by chromatographic methods were met by the puzzling finding that all five of their nitrogen containing fractions possessed activity and were more or less quantitatively the same Unfortunately Handler's only test of activity was the capacity to produce hypercalcaemia There have been repeated suggestions that more than one hormone is produced by the parathyroids, but to date, evidence for this has certainly been far from conclusive

Perhaps one of the complicating factors in this regard has been made visible by the intravenous calcium experiments mentioned previously From these it would appear that hypercalcaemia *per se* causes a rise in serum phosphorus, totally apart from its effect in "turning off" the parathyroid glands, for quantitatively the reduced urinary phosphorus in the normal person is inadequate to account for the rise in extracellular water phosphorus (Howard, Hopkins and Connor, 1953) furthermore, serum phosphorus rises under the stimulus of serum calcium elevation even in hypoparathyroid

patients and in other circumstances where phosphorus diuresis is coincident It appears that elevation of serum calcium, which is one action of parathyroid hormone, tends to raise serum phosphorus, whereas another action of parathyroid hormone tends to depress it Thus parathyroid secretion has a double effect on serum phosphorus—one results in increase, the other in decrease, the latter of course is the stronger force in the long run

Some Thoughts on the Mechanism of Calcium Homeostasis and the Rôle of the Parathyroid in it

The stability of the serum calcium concentration in normal persons has been previously mentioned Negative calcium balance can be withstood for long periods without significant hypocalcaemia, as witnessed by diarrhoeas and by lactating Chinese mothers (Liu *et al*, 1940) Rats have survived for several generations on minimal quantities of dietary calcium, each succeeding generation having more rarefied bones but without sufficient hypocalcaemia to prevent survival Huggins' and Hastings' (1933) dogs, bled at five minute intervals and replaced with calcium free blood, lost more calcium over a two hour period than was initially present in their entire extracellular compartment, yet soon after the experiment ended were *normocalcaemic*

Studies on the carcass of normal man indicate that, of some 1,200 g of calcium present, all but 12 g is resident in the skeleton (Mitchell *et al*, 1945) Thus from purely quantitative calculation any long term deficit must be made up from the bones if calcium concentration of the serum is to be maintained Calcium of non skeletal tissues, which can be calculated to be approximately 7 mg per 100 ml of intra cellular water, can hardly be visualized as participating to any extent in homeostasis since radioactive calcium studies have shown exchange to be minimal, and furthermore many important enzyme systems are inhibited by small concentrations of ionic calcium (Lehninger, 1950), indicating that most

intracellular calcium is probably "bound" In states of deficit, therefore, the calcium of the bones must be the source of homeostatic maintenance (Howard, 1953) Histological evidence has disclosed losses from the skeleton after heavy calcium deficit, and it is from the newest or most recently formed bone that the loss has occurred

It has previously been pointed out (Howard, 1953) that the kidneys and intestinal tract play relatively passive and inconspicuous rôles in calcium homeostasis, when compared with the bones This is not to decry or minimize the great losses that can result from these structures in diarrhoea or acidosis, the point is that in preserving the stability of concentration of calcium in body fluids their capacities are relatively limited and slow as compared with those of the skeleton's crystalline surfaces

The bones are admirably adapted anatomically for functional service in homeostasis, for it has been estimated that the trabeculae (Hendricks and Hill, 1951) provide acres of surface in contact with extracellular fluid, where forces capable of changing the movement of calcium to or from them could operate There is no need here to reduplicate comments on the structural characteristics of bone, but it is worth a reminder that it is a living tissue, that the youngest (newest formed) crystals are smaller and that the water content is greater than that of more mature bone (Robinson and Watson, 1953) The crystal elements have been shown to be in constant exchange with those of the extracellular fluids and recrystallization may be a normal feature of skeletal metabolism (Neuman and Neuman, 1953) From both histological and clinical evidence it is clear that the metabolism of bone is subject to a variety of influences Physical forces affect basic skeletal processes, the lumberjack's bones are thicker and radiographically denser than those of the sedentary worker The *lack* of such physical forces is even more strikingly manifest in the rapid loss of size and density of a bone when put at absolute rest, in a cast or during paralysis from neural injury

The growth and resorptive mechanisms in bone are subject also to influence by nutritive factors both local and systemic During periods of rapid growth of bone and also in rickets, where the extracellular fluids have a reduced concentration of phosphorus, calcium, or both, the presence of matrix not yet clothed with crystals is readily visible to the microscopist But during even the most rapid resorption from whatever cause, one does not see matrix, i e osteoid, from which the crystals have been removed (Park, 1954) Apparently nature's method of resorption is to "dissolve" simultaneously both matrix and crystals, whereas in the build up process, matrix clearly antedates the crystal agglomeration

It has been previously pointed out that in times of deficit the calcium of the skeleton meets the homeostatic need, and it is probably also the skeletal surfaces which are the major acceptors of calcium in times of an overabundant supply to the circulation If these premises are granted, one cannot then escape the fact that it is the overall relationship between the crystals of the bone and the calcium of the extracellular fluids which is the major determinant of the serum calcium concentration The homeostatic mechanisms which control serum calcium must operate then primarily at this interface If serum calcium is moving into the bones at a faster rate than its exit (and it is to be recalled that histologically bones never look everywhere to be in the same state of activity), and this difference is not made up by that which enters the system from the gut—then serum calcium will fall When more calcium is moving from the bones than to them, and the excess loss is not met by increased loss through the kidneys or intestines, then serum calcium will rise Thus when hyper or hypocalcaemia exist there is either a change in the capacity of the skeletal structures to respond to normal stimuli or one or more forces which constitute the normal homeostatic mechanisms have gone awry The secretion of the parathyroid glands is an important *one* of these homeostatic forces, and hence must carry out this function at the skeletal extracellular fluid interface

It was previously emphasized that removal of the parathyroid results in a prompt fall in serum calcium No coincident removal of calcium from the blood by way of urine or stool has been observed Loss of parathyroid hormone has suddenly changed the relationship between interstitial fluid and skeletal calcium in such a way that serum calcium is now 3 to 5 mg lower than its normal status, and, more interesting still, the fundamental relationship between the two phases has not changed, only the superficial relationship has altered For, in hypoparathyroidism a drain upon the serum calcium such as removal of blood and replacement with calcium free blood is still met by a prompt restoration of the serum calcium level, but now only to its subnormal point Contrariwise, injection of calcium salts is soon followed by return to the same hypocalcaemic level when administration of the calcium ceases There is still a basic relationship existing between the skeletal tissue salts and the serum calcium, absence of the parathyroids has altered it only to a degree In hyperparathyroidism, where an adenoma is hypersecreting presumably at a fairly fixed rate, one can likewise further elevate the serum calcium by injection of calcium salts, only to find prompt return to the previous hypercalcaemic level when the procedure is stopped

Hypothetical Deductions

From these data just recited, together with the many other types of environmental changes, both local and general, which can alter the relationship between the skeletal crystals and the extracellular fluids, it is difficult to escape the concept that this relationship is guarded or supervised by a living mechanism, and that it is upon such a biological system that the parathyroid hormone among other forces acts One visualizes therefore, a living barrier between the interstitial fluid and the tissues of the bone where the lime salts are resident And across this barrier there would necessarily be a gradient—a higher propensity to crystallization on the inside, greater solubility on the water or extracellular water side

The tendency for movement of the elements controlled by the gradient would be in one or the other direction, depending upon the height of the gradient at the given place and moment

This picturization is purely a conceptual one but has served, for us at least, as a useful working hypothesis to explain presently known facts and as an idea for future experimental testing From the histological point of view an actual membrane can be postulated as existing between bone crystals and interstitial fluid, composed of osteoblasts and protoplasmic strands between them, not unlike the endothelial layers of vascular channels Trabeculae, and even the inner surfaces of compact bone, would thus be pictured as encased, as a sausage in its skin, by what Dr Park has described as "the periosteum of the trabeculae" *With such an idea the parathyroid* hormone and other forces are viewed as directly affecting cellular function

A summation of this concept might be made as follows the bones have been shown, beyond reasonable doubt, to be the site of operation for a buffer system which stabilizes the concentration of calcium in the body fluids One conceives of the bone crystals as a gigantic pile of mineral materials, controlled locally by an active barrier, this barrier having an inherent or basic level of operation The parathyroid hormone is one force which affects it and alters its basic rate Structurally the barrier could be a membrane derived from endosteal cells or their proliferatives, and, therefore, changes in its properties would be due to cellular activity of the bone cells

In this visualization the bone cell has been given still further duties—those of the porter and the janitor, in addition to being the architect, the builder and the demolition squad of the skeletal mansion

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DISCUSSION

Dixon I have been struck for many years by the difficulties and confusion that arise when one tries to correlate the effects of parathyroidectomy and parathyroid grafts and the injection of parathormone and the variation among different species I have always felt that it is due to the fact that when we make the parathyroid extracts we break down the hormone as it exists *in vivo* Usually we extract by boiling the tissue with dilute acid which seems a very violent procedure Many years ago I was making some parathyroid extracts by this acid extraction of the parathyroid tissue and I happened to get the acid concentration wrong When I tested the extract by injecting it into dogs after 48 hours nothing happened but after 96 hours the blood Ca in the same dogs was up to about 30 which should not have been so That was a delayed action because I had got the acid wrong and I had not broken down the hormone to the small fragments that we normally get in commercial parathyroid extracts, which shows that the extract we use is by no means the natural parathyroid hormone

Howard I agree with that But I think it is fair to conclude that when we use those extracts we get effects equivalent to those which are produced by the spontaneous occurrence of hyperparathyroidism We get osteitis fibrosa in the animal we get hypercalcaemia hypophosphataemia and the same renal changes in both situations When we give this extract to the human who is hypoparathyroid in proper dose and before the antibody formation takes place we can biochemically at least reverse all the changes due to hypoparathyroidism Admittedly this is a crude extract but we cannot say that it does not induce at least many changes identical with changes from the hormone that is produced by the normal gland

Dixon The rat is particularly resistant to parathyroid extract and although parathyroidectomy produces its usual drop in serum Ca and grafts restore the serum Ca to normal parathyroid extract as we make it does not I think there is a fundamental difference

Nordin No that cannot be right there is a perfectly good assay based exactly on this in parathyroidectomized rats

Dixon You have to use comparatively large doses about one hundred times the physiological amount even though you use the parathyroidectomized rat

Howard The rat is less dependent on its parathyroid hormone than the dog or man If you parathyroidectomize the rat and give it a little Ca in the diet it does not usually go into tetany whereas the dog and man do

Follis If you tide the rat over the postoperative phase he can stabilize himself in a little while

Nassim Dr Howard do you think this endosteal membrane of yours is in a case of severe hyperparathyroidism destroyed in most linings of the trabeculae?

Howard That is the most difficult situation of all in which to detect a membrane histologically

Nassim Yet as soon as you remove the adenoma back comes your homeostasis and you have got an immediate effect

Lisco What makes you think that it is gone?

Nassim When you look for it are the trabeculae not being actively destroyed?

Lisco Have people really tried to look for it?

Howard You can still guess that there might be strands along there enzymatic action was on the destructive side It was reversing itself and being a demolition agent rather than a construction agent But the membrane might perfectly well still be there it must be there

Lisco I should think it must be there

Kodicek Your postulate about a membrane, or rather your postulate of a physicochemical mechanism is most welcome to me It would very nicely support the fact that there are quite a number of factors which are involved in the mineral turnover AT10 six vitamins D parathyroid hormone This low specificity would be explained if a physicochemical mechanism were operating When you then go on to a histological basis to try to show a membrane, I am not with you The membrane may come in at a cellular level that is it would be the function of the cells whatever function they have in producing the organic matrix on which mineralization then occurs more or less by physical factors governed by physical laws

Howard I think that 'membrane' may be the wrong word but that there must be a gradient across this barrier Physical factors seem to influence it too the crystals and matrix are protected from the outside physical factors but the barrier is also influenced by those outside factors

Kodicek You still maintain that there is a membrane around the bone?

Howard I do not say what kind of a membrane it is but something is different on one side where the crystal exists and on the outside On several occasions when we were doing *in vitro* experiments on calcification of cartilage we did not stopper the bottle and just shook it up with an ultrafiltrate of serum and there was a precipitate at the bottom of the bottle in the morning All that happened was that the pH changed to 8 6 so that in normal serum ultrafiltrate we have an apatite precipitation at pH 8 6

Kodicek I still have one point when you implant say bladder mucosa in muscle or liver you get ectopic bone formed I have seen no sign of a membrane there and it is proper bone

Howard You do not see a membrane in the cartilage

Kodicek I am not against the idea of a membrane except at this gross level

Howard I called it a barrier

Lisco I have no thesis to defend in this discussion about the membrane but I wonder whether the following observation might help to clarify the situation Suppose this is a trabecula of bone and suppose we inject an element such as ^{89}Sr or ^{45}Ca soon after injection we will find an accumulation of radioactivity in the mineralized portion of bone These

DISCUSSION

Dixon I have been struck for many years by the difficulties and confusion that arise when one tries to correlate the effects of parathyroidectomy and parathyroid grafts and the injection of parathormone and the variation among different species I have always felt that it is due to the fact that when we make the parathyroid extracts we break down the hormone as it exists *in vivo* Usually we extract by boiling the tissue with dilute acid which seems a very violent procedure Many years ago I was making some parathyroid extracts by this acid extraction of the parathyroid tissue and I happened to get the acid concentration wrong When I tested the extract by injecting it into dogs after 48 hours nothing happened, but after 96 hours the blood Ca in the same dogs was up to about 30 which should not have been so That was a delayed action because I had got the acid wrong and I had not broken down the hormone to the small fragments that we normally get in commercial parathyroid extracts which shows that the extract we use is by no means the natural parathyroid hormone

Howard I agree with that But I think it is fair to conclude that when we use those extracts we get effects equivalent to those which are produced by the spontaneous occurrence of hyperparathyroidism We get osteitis fibrosa in the animal we get hypercalcaemia hypophosphataemia and the same renal changes in both situations When we give this extract to the human who is hypoparathyroid, in proper dose and before the antibody formation takes place we can biochemically at least reverse all the changes due to hypoparathyroidism Admittedly this is a crude extract but we cannot say that it does not induce at least many changes identical with changes from the hormone that is produced by the normal gland

Dixon The rat is particularly resistant to parathyroid extract and although parathyroidectomy produces its usual drop in serum Ca and grafts restore the serum Ca to normal parathyroid extract as we make it does not I think there is a fundamental difference

Nordin No that cannot be right there is a perfectly good assay based exactly on this in parathyroidectomized rats

Dixon You have to use comparatively large doses about one hundred times the physiological amount even though you use the parathyroidectomized rat

Howard The rat is less dependent on its parathyroid hormone than the dog or man If you parathyroidectomize the rat and give it a little Ca in the diet it does not usually go into tetany whereas the dog and man do

Follis If you tide the rat over the postoperative phase he can stabilize himself in a little while

Nassim Dr Howard do you think this endosteal membrane of yours is in a case of severe hyperparathyroidism destroyed in most linings of the trabeculae?

Howard That is the most difficult situation of all in which to detect a membrane histologically

port as it were, and the other school which says that it acts on bone and that the result of the action on bone is the high level Now it seems to me that Dr Howard has offered us the opportunity of bringing together these two conflicting ideas, and might I interpret his views as suggesting that according to the amount of circulating parathyroid hormone the mineral will come off bone into the circulation at different levels If there is very little parathyroid hormone or none there it will not come off until the Ca goes down, to below 7 for instance and if there is a lot circulating it comes off until the level is say 10 In other words in the presence of a parathyroid tumour with a circulating Ca of 10 if you pumped in enough Ca intravenously to maintain that level artificially you should be able to stop the Ca coming off the bone Is that right?

Howard I would so conceive it

Blaxter If you regard the serum Ca as homeostatically maintained that must represent a steady state between bone the small uptake from muscle tissue and renal excretion Those processes, from what is known of Ca metabolism are rate control processes They are reversible and if those rates are rapid then you would get low levels of serum Ca I think there is no need to postulate any membrane whatever One could say quite simply that the diffusion pressure of the extracellular fluid in the bone substance changes

Howard How would the parathyroid hormone manufacture such a thing in an hour or two?

Blaxter That I do not know

Howard Well it affects the rate and the rate is an enzyme system ' Membrane ' may be the wrong word to use the fact is that you have got a fundamental rate and you affect that rate up or down

Van Slyke Dr Howard mentioned the fact that kidneys play rather a small rôle in the serum Ca concentration which is governed chiefly by equilibrium between bone and serum Some of the cases of nephrosis that we have had at the Rockfeller Hospital entirely support this concept We have had such cases with only 6 mg of serum Ca consistently, and when we examined the urine they were excreting only a few mg per day The kidney tubules were reabsorbing Ca completely but equilibrium with the bones still gave the low serum Ca that is associated with a lack of parathyroid hormone The low serum Ca was not caused by loss through the kidneys but occurred in spite of almost zero excretion

substances are soluble and they readily enter into the mineral metabolism of the trabecula On the other hand if you inject an element like ^{239}Pu, you find that it is deposited alongside the trabecula as if separated from bone by some endosteal barrier which it is unable to penetrate Might there not be a membrane like structure in this region?

Engstrom I think other membranes in the organism are highly organized lipoprotein structures What Dr Lisco said is not surprising that very young structures take up isotopes very quickly and that old ones do not take up isotopes at all Generally when you give large doses of parathyroid hormones you will get a great number of resorption cavities both in young and in old structures and these resorption cavities are often lined with a very thin layer of lowly mineralized tissue and these layers have a large uptake of isotopes

Lacroix Dr Howard did not state explicitly that there was a dual mechanism in Ca homeostasis but it seems to me that it was implicit in his presentation I feel from the anatomical standpoint that there is a dual mechanism one which would account for the 7 mg /10 mg which you have mentioned and another which would account for the other 3 mg the first mechanism being an equilibrium between part of the bone and the blood and the second being accounted for by the action of parathormone

Howard Let us talk of it in terms of interstitial fluid and say that it starts at 6 5 or 7 and that in the parathyroidectomized animal it is down to 4 in the extracellular water Now that is a basic relationship If you want to talk about it in terms of whole blood serum let us talk about it in terms of ten and six ten ordinarily and six in parathyroid ectomy

Lacroix What is your opinion about this basic mechanism? Is it an equilibrium?

Howard Yes I think it has got to be because when you add or detract Ca from the blood this same mechanism must support it

Lacroix Do you think that it is an equilibrium with the whole of the skeleton or with part of the skeleton?

Howard I think various parts of the skeleton may react to it more or less inside of the bone less the outside reacts more simply because an influencing agent has the capacity anatomically to get there and work faster

Nordin I would like to thank Dr Howard for what I think is a tremendously helpful concept Could we not imagine however that this is a theoretical membrane and that it exists at the sort of crystalline level that Engstrom discussed? It seems to me that the problem of the discussion is that the dimensions of the membrane you can actually see —that Dr Howard mentions—are of the order of 2 or 3 μ whereas the level at which the thing is actually taking place as Engstrom shows is a matter of Å It is a different world Surely if there is a membrane it is not going to be at this big level but is only a theoretical membrane

The second point I would like to make is that there has always been a conflict between the amount of evidence which suggests that the para thyroid hormone regulates the actual level of circulating Ca the trans

deviation of the clearance ratio along this line is 0 06 Fig 2 shows the relevant part of Milne, Stanbury and Thompson's chart, with the mean regression line and the 95 per cent limits drawn in by us

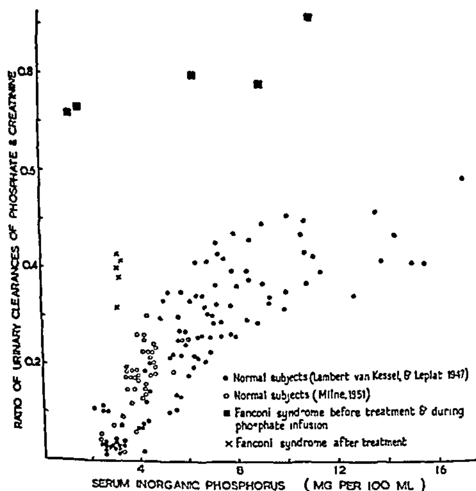


FIG 1 The relationship of the phosphate/creatinine clearance ratio to the serum phosphate level as derived from phosphate infusions by Milne Stanbury and Thompson (1952) and Lambert van Kessel and Leplat (1947)

We now turn to our own observations We have measured the phosphate/creatinine clearance ratios in a number of different conditions, and in Fig 3 we show the results obtained in 16 cases of osteoporosis, 10 cases of osteomalacia, 4 mild

THE INDIRECT ASSESSMENT OF PARATHYROID FUNCTION

B E C Nordin and Russell Fraser
Postgraduate Medical School of London

The phosphate diuretic action of parathormone is not easy to measure, for even when phosphate excretion is expressed as a clearance this clearance still varies with the serum phosphorus level The object of this paper is to show that changes in the activity of the parathyroid glands are associated with changes in phosphate clearance and how these are related to the serum phosphate concentration

Milne, Stanbury and Thompson (1952) have already shown that when the serum phosphate is raised by the infusion of inorganic phosphate solutions there is, in normal subjects, a rise in the ratio of the phosphate clearance to the creatinine clearance (C_p/C_{cr}) They have assembled their data and those of Lambert, van Kessel and Leplat (1947) into a composite chart (Fig 1) which shows the normal relationship between the serum and urinary phosphate We have selected all the points on this composite chart up to a serum phosphate level of 8 mg per 100 ml and have taken the liberty of assuming that the regression in this part of the chart does not depart significantly from linearity On this assumption, we have calculated the regression equation of these 117 points and found that it can be expressed as follows

$$C_p/C_{cr} = \frac{\text{Serum P}}{20} - 0\ 05$$

In other words for each rise of 1 mg per cent in the serum phosphate level the clearance ratio rises 0 05, and the regression line intercepts the C_p/C_{cr} axis at $-0\ 05$ The standard

clearance ratio that would be expected normally for the serum phosphate level from the observed value This can be done

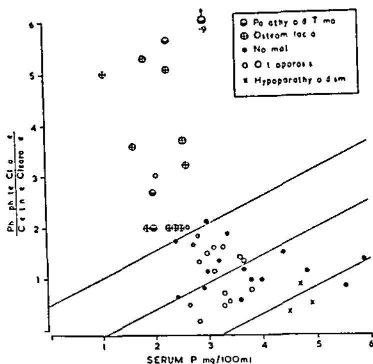


FIG 3 Relationship of the phosphate/creatinine clearance ratio to the serum phosphate level in normal subjects and in patients with osteoporosis osteomalacia hyperpara thyroidism and hypo parathyroidism (authors cases) Regression line and 95 per cent limits as in Fig 2

with the help of the regression equation, since in normal people the following equation should hold

$$C_p/C_{cr} - \frac{P}{20} = -0\ 05 \pm 0\ 12$$

$$\text{or } C_p/C_{cr} - \frac{P}{20} + 0\ 05 = 0 \pm 0\ 12$$

When this is done, the results shown in Fig 4 are obtained We have called this measure of the abnormality of the C_p/C_{cr}

cases of hypoparathyroidism, 4 cases of parathyroid tumour and 24 normal subjects It will be seen that the data on our normal and osteoporotic subjects (with one exception) lie within, or on the edge of, the 95 per cent limits which we have calculated from Milne, Stanbury and Thompson's data

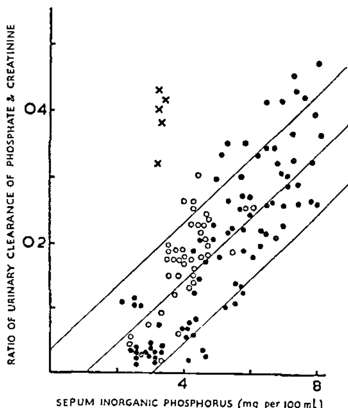


FIG 2 All data from Fig 1 up to a serum phosphate level of 8 mg /100 ml The mean regression line and the 95 per cent limits have been calculated by the present authors

All the cases of osteomalacia and primary hyperparathyroidism have higher clearance ratios with low serum phosphate levels, and 3 of the hypoparathyroid cases have abnormally low clearance ratios with high serum phosphate levels

Our next step has been to calculate the departure of each point's C_p/C_{cr} from the normal mean line by subtracting the

The high values in Cushing's syndrome are of interest and suggest that the low serum phosphate of this condition is associated with impaired tubular reabsorption of phosphorus Could this be due to potassium depletion?

Now I wish to turn aside for a moment to remind you of the effect of intravenous calcium on phosphate excretion (Howard,

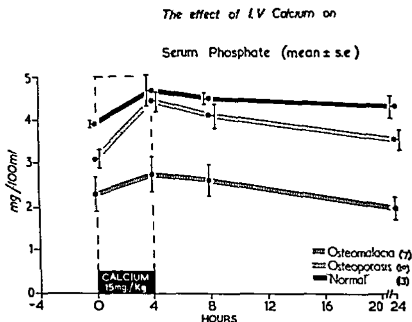


FIG 5a

A 4 hour infusion of calcium gluconate is associated with a rise in serum phosphate level (5a) followed by a steady fall in phosphate/creatinine clearance ratio (5b) p 228

Hopkins and Connor, 1953) We have already published our findings on this subject (Nordin and Fraser, 1954) and Figs 5a and 5b are taken from our previous paper They show that a 4 hour infusion of calcium gluconate (15 mg Ca per kg body weight) is followed by an immediate rise in serum phosphate and a progressive fall in C_p/C_{cr} the latter reaching its lowest point about 12 hours after the commencement of the infusion We have already reported our reasons for believing that these reverse changes in serum and urine phosphate

(and so of renal phosphate excretion) the "Phosphate Excretion Index" (P E I) A figure above + 0 12 or below − 0 12 should then signify an abnormal phosphate/creatinine clearance ratio, irrespective of the *serum phosphate level, and it* will be seen that this single figure separates patients with abnormal parathyroid function from normal subjects The

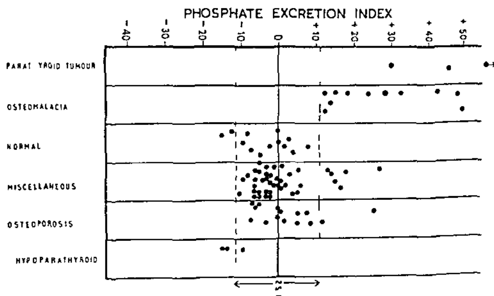


FIG 4 The mean expected phosphate/creatinine clearance ratio for the blood level is defined as zero The normal range embraces two standard deviations above and below zero (i e − 0 12 to + 0 12)

10 cases of osteomalacia (with presumed secondary hyperparathyroidism) and the 4 of parathyroid tumour all lie above the normal range and 3 of the *hypoparathyroid cases fall below* it Most of the 'miscellaneous' cases fall within the normal range, but there are 9 above it made up as follows

Cushing s syndrome	4
Renal stone	2
Vitamin D intoxication	1
? Parathyroid tumour	1
Myxoedema	1

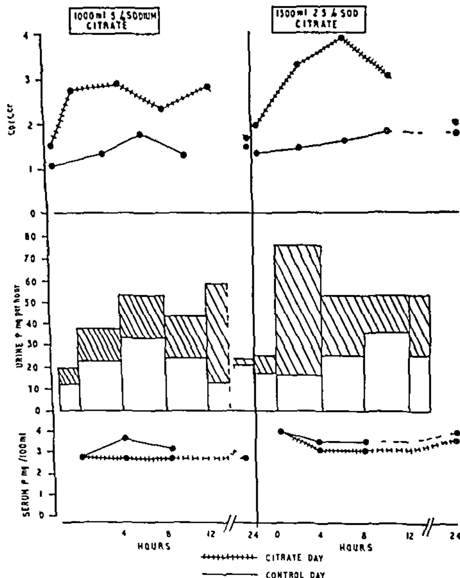


FIG 6 Two 8 hour infusions of sodium citrate in normal subjects In each case the serum phosphate urine phosphate output and phosphate/creatinine clearance ratio on the control day and the infusion day are shown The differences in serum level may not be significant but infusion of citrate is followed by a marked rise in phosphate output per hour and in the phosphate/creatinine clearance ratio

animals leads to a rise in serum calcium which is believed to be due to stimulation of the parathyroids, but to the best of our

are due to suppression of the parathyroid glands by the elevated serum calcium As Dr Howard has just stated, there is a good deal of evidence that the activity of the parathyroid glands is controlled by the concentration of calcium in the body fluids, presumably it is, in fact, the concentration of

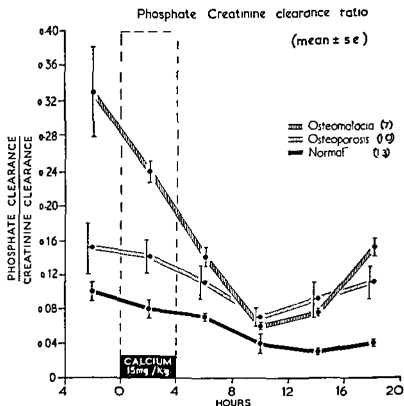


FIG 5b

ionized calcium which is the important factor Since elevation of the serum calcium apparently leads to suppression of parathormone secretion, we thought that reduction of ionized calcium by infusion of sodium citrate might have the opposite effect Other workers (Stewart and Bowen, 1952) have already shown that the injection of oxalate and citrate salts into

the serum phosphate, the urine phosphate in mg per hour, and the C_p/C_{cr} are shown The slight differences between the serum phosphate levels on the control days and on the citrate days are probably not significant, but in each subject there was a substantial rise in total phosphate output and in C_p/C_{cr}

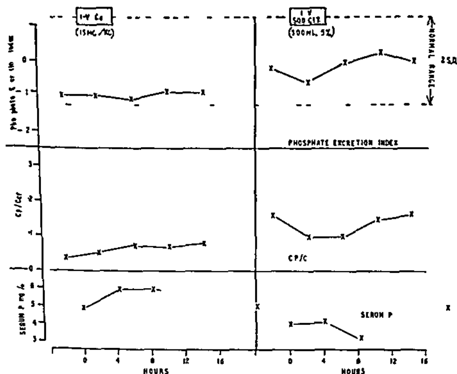


FIG 8 The effect of calcium gluconate and sodium citrate infusions in a patient with hypoparathyroidism Neither infusion was associated with the reverse changes in serum and urine phosphate which occurred in normal subjects

following the citrate infusion A little tingling around the mouth was the only sign that the ionized calcium level was in fact being reduced

In Fig 7 we have compared the effect of calcium gluconate and sodium citrate infusions on the Phosphate Excretion Index with the diurnal changes seen in three normal subjects without any infusion This method of presentation shows

knowledge no similar observations have been made in man Milne (1951) observed a rise in urine phosphate after infusion of sodium citrate, but he did not attribute it to parathyroid gland stimulation

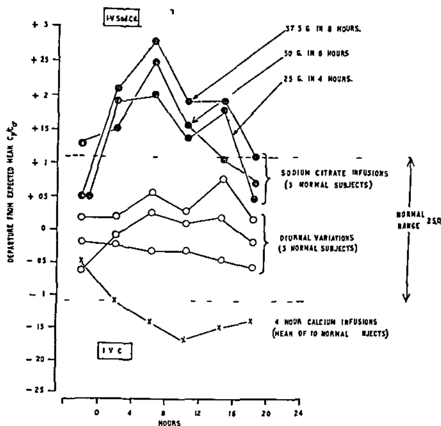


FIG 7 A comparison of the effect of calcium gluconate and sodium citrate infusions on the phosphate excretion index By contrast the diurnal variations in 3 normal subjects do not extend outside the normal range

We started by infusing fairly small amounts of sodium citrate (12 5 g in 4 hours) and observed a slight phosphate diuretic effect As the dose was increased the effect became more marked and the result of two 8 hour infusions (37 6 and 50 g respectively in 8 hours) are shown in Fig 6 In each case

phosphate described by Dr Nordin that were calculated by intravenous infusion when the blood was brought temporarily to a higher level than the tissues the phosphate then went two ways some went out cleared by the kidney and some went into the water of the other tissues The picture was not quite so clear as if you calculated the clearance with everything absolutely constant on a fixed diet We have had patients who have been for experimental purposes on widely varying phosphate intakes on which the blood levels of phosphate were not materially affected and yet there were very wide variations indeed in the phosphate clearances when calculated from 24 hour urine phosphate divided by the blood level giving ml /min of phosphate cleared By doubling up the phosphate intake for instance you could get an apparent doubling up of the phosphate clearance and yet the blood level remained constant The actual glomerular filtration rate was checked now and again by inulin clearances not by creatinine clearances It is very difficult to estimate plasma creatinine at the ordinary endogenous level The uptake is so low that it is difficult to get any accurate creatinine clearance Have you measured plasma creatinine in your calculations on clearances ?

Nordin This is a basic point I do not feel that you can call a 24 hour collection with the blood taken some time during 24 hours a clearance The whole essence of the clearance phenomenon is that you take a period of time you take your blood in the middle of that, and you assume that your blood level is not changing We know that there are big diurnal variations in the phosphate and I consider that even 4 hour clearance is a bit too long to be quite right We now do the base line clearance in the morning which is our standard We do that on a 2 hour basis and I think 2 hours is about as long as you can reasonably do a clearance on a blood level which you know is fluctuating So I would be reluctant to call those data of yours clearances We have looked into the question of the effect of diet and have given a normal subject varying amounts of P in the diet The serum phosphate level varied from 2 5 to 4 5 and as it did so the C_p/C_{cr} went up as predicted so that all the observations were in the normal range of Milne Stanbury and Thompson You cannot talk of a phosphate clearance without simultaneously referring to the serum phosphate level I do not think that one observation of serum phosphate in 24 hours can meet that point

Dixon If you give diets high in phosphate you will not materially affect the serum phosphate levels but you will shoot out enormously increased amounts of phosphate That means your phosphate clearances have gone up

Nordin The point is that you do change the serum phosphate level

Dixon Not so markedly as the increase in urine phosphate You might alter it by 10 or 20 per cent but not more

Dent That depends on the slope of his curve

Nordin As your blood level goes up you divide by that blood level but phosphate excretion goes up so much that the clearance rises too As far as the blood creatinine is concerned I agree I think it is a very tricky thing and I wish we did not have to estimate it but methods are

more clearly the significance of the changes that are occurring, and demonstrates that calcium infusion reduces the C_p/C_{cr} to definitely subnormal levels, while citrate infusion raises it into the hyperparathyroid range By contrast, the diurnal variations in C_p/C_{cr} and serum phosphate level in 3 normal subjects are related in such a way that the P E I remains within the normal range throughout

It is difficult to prove that the effects we have described are due to changes in parathyroid activity, but their failure to occur in hypoparathyroidism offers some support for this hypothesis Fig 8 shows the effect of calcium gluconate and sodium citrate infusions on the serum phosphate, C_p/C_{cr} and P E I in a case of postoperative hypoparathyroidism The case was a mild one and was fairly well controlled on calciferol when the citrate infusion was performed However, it is clear that no significant change occurred in the tubular reabsorption of phosphate after either calcium or citrate infusion

In conclusion, our observations suggest that abnormalities in renal tubular reabsorption of phosphate, including variations in parathyroid activity may conveniently be expressed by the Phosphate Excretion Index

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DISCUSSION

Dixon At Stanmore we calculated our phosphate clearances by reference to a 24 hour period when a patient was in the metabolic ward on an *absolutely constant intake so that all the tissues blood and* urine were in equilibrium as it were I felt that in the clearances of

Nordin No it might have been so, but so far it has not turned out like that They are all over the normal range, and the mean and standard deviation of the osteoporotics and the normals calculated independently are the same

Nassim My colleagues Drs P D Saville and Lily Watling and I have studied this As is well recognized the serum phosphate in post menopausal osteoporosis tends to be high normal This we feel may be due to an anterior pituitary effect When patients are treated with stilboestrol the serum phosphate tends to drop Phosphate clearances

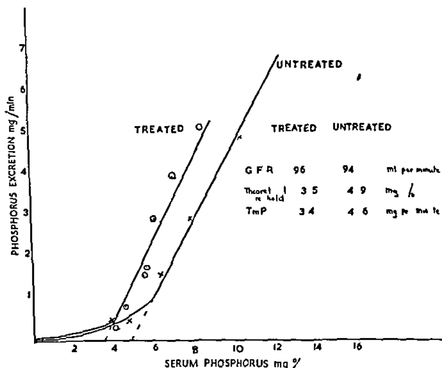


FIG 2 (Nassim)

have been performed on three women using the technique of Anderson (1955 *J Physiol* **130** 268) All three studies show clearly the increased phosphate clearance after administration of stilboestrol (Figs 1–3)

Nordin It is not easy to convert these figures straight over this is phosphate excretion in mg /min It requires a bit of mathematics to convert that to a clearance ratio I think there is obviously a slight change in tubular reabsorption but I suspect that the biggest change is in the blood level and that the consequent change in tubular reabsorption leaves them both still within the normal range Do you think that is possible?

Dent Yes, there is such a wide range

getting better and I think we are accurate within 10 per cent in our blood creatinine levels We have done a lot of duplicates and triplicates at different times of the day and they certainly are within 10 per cent if done by Roscoe s method There are other methods which we hope to use But I think even if you take into account the errors in the blood creatinine level the differences that I have shown you remain highly significant

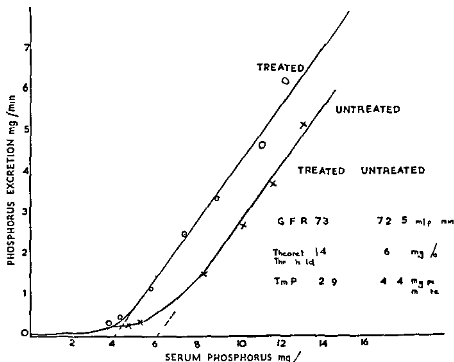


FIG 1 (Nassim)

Figs 1 2 and 3 show studies of phosphate clearance in 3 women before and after treatment with stilboestrol

Points to be noted are that on each occasion there has been no change in glomerular infiltration and that after treatment the slope has been shifted to the left showing lowering of tubular phosphate re absorption

In Fig 3 the clearance was done on stilboestrol first and a fortnight after withdrawal and therefore the oestrogen effect is probably persisting

Nassim Were the osteoporotics which you showed in Fig 5 post menopausal women?

Nordin Most of them were There are four cases of what we call pregnancy osteoporosis and Dr Dent calls idiopathic osteoporosis included in that

Nassim It seems to me that those levels were getting on the lower side of normal towards the hypoparathyroid level

Nordin Yes, I suppose that is really a better term than the term phosphate excretion index' I do not know if one can calculate the clearance ratio by radio-isotopic dilution techniques

Nicolaysen Did you measure the citrate output in the urine?

Nordin No we did not measure the citrate output in the urine we measured the blood citrate level in two of them It went up to 12 mg per cent in one and 17 mg per cent in the other

Nicolaysen Alkali ingestion may have an influence

Nordin I have tried bicarbonate and if bicarbonate does anything it does the opposite it puts down the serum phosphate and the urine phosphate at the same time As far as the base is concerned our standard dose is now 500 ml of 5 per cent sodium citrate given in four hours that is about 100 m equiv

Nicolaysen Are you sure that your creatinine clearance is not influenced by this difference?

Nordin Dr Dent raised this question on our Ca infusion work, so we did a few inulin clearances at that time but they did not make any very useful contribution We have published (Nordin B E C, and Fraser, R (1954) *Clin Sci* 13 477) the creatinine output per hour after Ca infusions and we think it is unchanged although there is a tendency for people to put out slightly less creatinine in the night than they do in the day I have not worked out the same thing after citrate infusions but it would have to be a very big rise in GFR to account for this sort of rise in phosphate output

Blaxter If you give the kidney more phosphorylating work to do that is if the kidney has to do more in the way of taking up inorganic phosphate for phosphorylating purposes does that change your clearance ratio and can that account for the effect of citrate?

Nordin Why does it have to take up more? The citrate has not put the serum phosphate level up We are not using anything that influences the phosphate level itself As I showed you the serum phosphate on the citrate day is a little lower than the serum phosphate on the control day So you would expect that the urine clearance should be lower if anything but in fact the urine clearance is always higher

Blaxter I was thinking of the phosphorylation in the wall of the tubule on reabsorption

Nordin But why should there be a change in phosphorylation if there is no change in blood level of phosphate?

Blaxter That is a systemic blood level isn t it? It tells one little about the phosphate levels in the renal cells

Armstrong May I put a general question that has to do with the subject of mechanism of action of parathormone? I think we all are convinced that the parathyroid hormone does demonstrably have an effect directly on the bone and that it also alters phosphate reabsorption by the renal tubules The evidence for both of these points is very clear My question is how is it that a single substance can affect vastly different physiological processes in ways that produce the same result that is the mobilization of the skeletal elements? Can anyone point out to me an analogous circumstance in the case of some other substance?

Nassim There is no doubt about the fact that phosphate clearance is altered with stilboestrol

Follis Dr Nordin are your cases of osteomalacia histological or radiological?

Nordin The osteomalacias are absolutely water tight The osteoporoses are just what people at the moment ordinarily call osteoporoses

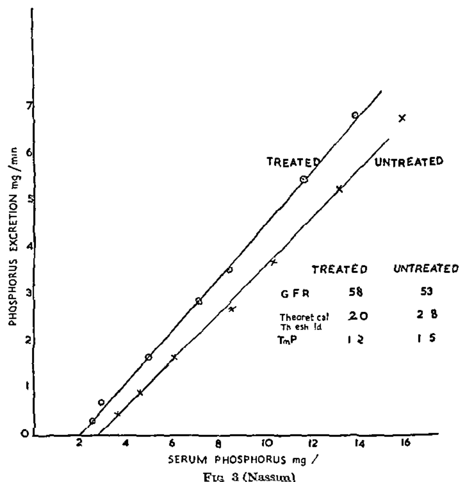


FIG 3 (Nassim)

But for the purposes of this presentation here they include what Dr Nassim calls grades I II and III In other words they are people whose bones look thin and who have got back ache and those who have cod fish vertebrae and those who have got crush fractures We try to confine ourselves to the ones with cod fish vertebrae and crush fractures

Kodicek Would it be possible to estimate the clearance ratio by isotopic dilution techniques or has this been done? Another point could you not call your index a variance?

VASCULARITY OF BONE IN RELATION TO PATHOLOGICAL STUDIES

(Paget's Disease, Sudeck's Atrophy, Osteoarthritis of the Hip)

E RUTISHAUSER

Institute of Pathology Geneva

AN examination of the circulation of blood in bone has confirmed the results obtained by a number of authors, i e

(1) the arterial supply is multiple,
(2) the capacity of the venous system is very large,
(3) the respective vessels of each kind of bone tissue (bone tissue proper haemopoietic bone marrow, fatty marrow) form an entity in the adult bone, which can be divided schematically into territories opening one into the other (Rutishauser, Rouiller and Veyrat, 1954)

Experience with epiphyseal necrosis has shown that insufficiency of the collateral circulation frequently occurs Three types of capillaries are found in bone marrow

(a) arterial capillaries, which are narrow and straight, and have few ramifications and which are less numerous than venous capillaries,
(b) peripheral venous sinuses (sinus of the third order), which are numerous, large, and have many anastomoses they form blood reservoirs in bone,
(c) narrow dormant capillaries, i e Doan's intersinusoidal capillaries which form a compact network at the cellular level (Doan 1922*a* and *b* 1923)

We think that this network is parallel to that of peripheral sinuses (see Fig 6 in Rutishauser, Rouiller and Veyrat, 1954)

Kodicek Vitamin D

Armstrong How?

Kodicek Again it is rather vague vaguer than parathormone but there is a suspicion that vitamin D exerts its influence not only on the intestine, but has also a local action in bone and possibly affects the reabsorption in the kidney tubules

Fanconi If Dr Nordin s paper is right we must avoid giving citrate in osteomalacia and on the contrary, we now give citrate in the treatment of osteomalacia

Nordin I do not believe that the oral administration of citrate causes anything like the rise in blood level which we are getting with our infusion

Nicolaysen I think you should differentiate very sharply between the solubilizing effect of citrate on Ca in the intestine and the postabsorptive effect



Fig 1 A 62/54—Osteoarthritis pressure area—M 84 yr H E × 12 Indian ink injection Hyperplasia of the vessels Stasis of the sinuses and plasmostasis

Fig 2 T 6568/5-—Osteoarthritis periphery of the pressure area—M 53 yr Mallory × 57 C On the left plasmostasis On the right the sinuses are filled with blood (venous congestion)

Rast has examined these sinusoidal structures by injecting Indian ink into the adipose tissue of the breast, into the adipose tissue of the human mesentery, and also into the mesentery of the rat (Rast, 1955) On a morphological basis, our observations have demonstrated that these capillaries are identical in all localizations of adipose tissue

The results obtained by other authors by means of electronic microscope examination give no further information on this particular point

Even if the morphological aspect seems identical there exist, however, considerable functional differences between intra osseous adipose tissue and the adipose tissue at other sites The most important of these differences is the rarity of the haemopoietic function in the extra osseous adipose tissue To cite another difference plasmostasis in the intersinusoidal capillaries of osteoarthritic bone marrow frequently occurs (Rutishauser *et al*, 1952, Rutishauser and Grasset, 1954)

Ratzenhofer's work (Ratzenhofer and Propst, 1953) raised the hope that the same phenomenon might appear in adipose tissue of the breast and that this phenomenon might initiate the sclerosis of this gland However, Rast (1955) did not definitely find either plasmostasis or Ratzenhofer's *Oedempfutzen* (Ratzenhofer and Propst, 1953) in adipose tissue of the breast but he observed the formation of pre cipitated hyalin after slight fibrocytic proliferation

The precise points to be discussed now are as follows

(a) arteriolar and venous hyperplasia in certain osteo pathies,
(b) plasmostasis in relation to fibrosis
(c) certain aspects of fibrosis

(a) Vascular hyperplasia in Paget's disease has been studied (Rutishauser, Veyrat and Rouiller, 1954) However, it is important to stress that while the physiological repercussions of this hyperplasia have been demonstrated in Paget's disease (Edholm, Howarth and McMichael, 1945, Edholm and Howarth, 1953, Schwiegk and Lang 1951), their precise

Fig 5 T 6568/52—Pressure area of osteoarthritic femoral head—M 53 yr Mallory × 57 6 Marked fibrosis and venous congestion in the pressure area

Fig 6 T 2576/53—Right osteoarthritic femoral head—F 66 yr H F × 77 Vascular proliferation in a mesh like form This territory is very close to a necrobiotic cyst in the pressure area

Fig 3 T 1325/47— Left astragalus—M 38 yr H E × 46 Dystrophic Stage II of the Causalgia of Sudeck presents reticulosis and plasmostasis Disappearance of adipose tissue and its replacement by dilated sinuses Vascular neoformations originating especially in the sinuses

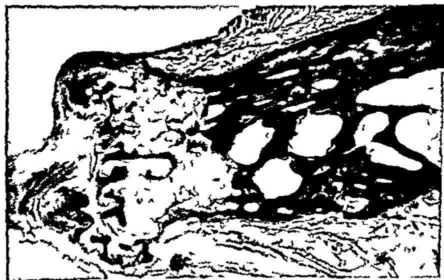


Fig 4 A 377/52—Middle phalanx of the little finger of the left hand—F 18 yr H E × 15 Atrophic Stage III of the Causalgia of Sudeck Specimen injected with Indian ink the vascularization is poor the phenomena of reticulosis have disappeared

nature in Sudeck's atrophy is unknown In osteoarthritis of the hip, Trueta and his associates (Harrison, Schajowicz and Trueta, 1953) think that this phenomenon has, as an end-result, an active hyperaemia, but we are inclined to believe that the end result is a venous congestion of the epiphysis (Rutishauser *et al*, 1952, Rutishauser and Grasset, 1954) Observations made during resection of osteorthritic femoral heads seem to support this point of view

(b) Morphological definition of plasmostasis a substance which stains like blood plasma and dilates the sinusoidal capillaries The injection of Indian ink into the bone marrow shows that intersinusoidal capillaries form a closed system Therefore, these must not be confused with Ratzenhofer's *Oedempfutzen*

In the intersinusoidal capillaries, three overlapping possibilities exist areas of plasma alone, areas of whole blood, and areas of plasma with occasional blood cells Plasmostasis is thus a particular functional state, localized in the intercellular system The physical and chemical qualities of this mass in the intersinusoidal capillaries which we believe to be blood plasma (purely on a morphological basis) have not been examined This plasmostasis is often found also in the region where the peripheral sinuses are dilated, but this relationship is not always evident Serial sections of these territories of stasis have not been made, and would, perhaps, lead to a better understanding of the relation of plasmostasis to sinus stasis Plasmostasis is a common phenomenon in the circulatory pathology of bone It has been observed in the pressure area of osteoarthritis of the hip and Stage II of Sudeck's disease but has not been definitively studied elsewhere (Figs 1 and 2)

It is necessary to examine the pathogenic rôle of plasmostasis in the proliferation of the reticulo endothelial cells Such an examination reveals, in certain cases, reticulosis and fibrosis of bone marrow

(c) Certain aspects of fibrosis

The cytological study of medullar fibrosis in bone is not

The data now presented concern these last three forms, none of which tend to develop hyalinosis

Paget's disease of bone in its active form is accompanied by important haemodynamic modifications, comparable to those produced by an arteriovenous fistula the vascular resistance of a Pagetic limb is diminished, its blood flow is augmented, the oxygen tension and the venous blood pressure are abnormally increased, as is the cutaneous temperature at the level of Pagetic bone In the active polyostotic stages the cardiac output is greatly increased (Edholm, Howarth and McMichael, 1945 , Edholm and Howarth, 1953, Schwiegk and Lang, 1951)

Comparison of healthy and Pagetic tibiae of the same subject shows an extraordinary growth in the number and calibre of blood vessels in the cortex and periosteum (Rutishauser, Veyrat and Rouiller, 1954)

The proportion of different types of blood vessels is also modified in the Pagetic bone arterioles and arterial capillaries are proportionally much more numerous In several regions a proliferation of arterial capillaries can be observed It was not possible to demonstrate a direct communication between the arteries and venous sinuses

The study of vascularization in a focus of osteoporosis circumscripta cranii—early stage of Paget's disease—permits the identification of the same vascular transformations and the recognition of their precocity, the first vascular transformations even precede the remodelling of bone

These important vascular transformations, which seem to explain the haemodynamic modifications in Paget's disease, are all reversible In the stabilized forms of Paget's disease—when the quantity of serum alkaline phosphatase is hardly augmented—vascularization of the bone is again normal (Rutishauser and Veyrat, 1954 Rutishauser, Veyrat and Rouiller, 1954)

Eleven cases of Sudeck's atrophy have been examined in the dystrophic Stage II of the disease, reticulosis was observed originating in Doan s intersinusoidal capillaries

easy, perhaps the decalcification of bone may render more difficult the use of the necessary stains, e.g. May Grunwald Giemsa's stain. Erdheim (1935–1936) has already mentioned the problem involved in cellular differentiation of the marrow in Paget's disease.

In order to demonstrate fibrosis of bone it is necessary to give it a proper place in the outline of fibrosis in general. Therefore, to reach an all-embracing definition is quite difficult.

The authors whom we have consulted do not define the nature and limits of fibrosis, sclerosis and hyalinosis (Anderson, 1953, Roulet, 1937, Ratzenhofer and Propst, 1953). The term "hyalin" applied to the connective tissue, is used to define a homogeneous tissue, which is more or less structureless, this term corresponds purely to an optic property and does not suggest the chemical composition of the modified tissue.

Some types of hyalinosis are of a metabolic nature. Amyloidosis is the only type of hyalinosis whose metabolic nature is certain. The others are apparently due to local causes.

(1) Keloid tissue is perhaps a hyalin modification.
(2) Fibroblastic hyalinosis, e.g., the tendon-like cords in Dupuytren's contracture (Rutishauser and Lagier, 1955).
(3) Hyalinosis in adipose tissue has been examined by Ratzenhofer and Propst (1953), and others. Rast (1955) has studied hyalinosis in the breast. Liposclerosis which is characterized by a necrobiotic component, must be considered separately (Blanc, 1951).

In the adipose tissue of bone marrow, there can be distinguished:

(a) Angiofibrosis in the active stage of Paget's disease.
(b) Angioreticulosis in Stage II of Sudeck's atrophy.
(c) Fibrosis with osteogenic potentiality developed in the pressure area of osteoarthritis.

original articular cartilage The superior layer is the seat of vascular resorption and ossification In the non pressure area where the cartilage is split into two layers, there is neither stasis nor vascular hyperplasia Fibrosis does not occur at this level, nevertheless, one can find areas of plasmostasis The intermediate area (area between the non pressure area and the pressure area) is relatively narrow It is thought that the vessels which split the cartilage into two layers are capsular in origin

Acknowledgement

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Proliferation of the arterioles and venulae was evident The venous sinuses were dilated (Fig 3) In the atrophic Stage III of the disease, the circulatory pattern has become normal again (Fig 4)

Osteoarthritis of the hip Figs 5 and 6 show a pressure area and a non pressure area in the pathological femoral head Vascularization of the osteophytes and splitting of the cartilage will not be discussed here (see Rutishauser and Grasset, 1954)

The pressure area is a region of bony sclerosis, of wear and tear of the cartilage, and contains cysts At this level the remodelling of bone reflects the active pathological progress of osteosclerosis There is an intense stasis, which can even produce haemorrhage However, the presence of hemosiderin is rare In the areas of plasmostasis two phenomena can be observed in the adipose tissue haemopoiesis is displaced by plasmostasis and fibrocytosis appears in its place The fibrils are coloured yellow by van Gieson's stain The presence of fuchsinophil fibres is exceptional Silver impregnation by the Tibor Pap method reveals a very loose reticular network This fibrosis is derived from the intercellular reticulo endothelial cells However, the sinusoidal wall contributes in part to this fibrosis since it originates in this region At a later stage the arterial adventitia may contribute to its formation This fibrosis replaces the adipose tissue Peripheral sinuses appear dilated and injection of Indian ink no longer identifies the sinuses This fibrosis has potentialities of undergoing either cartilaginous metaplasia or osseous metaplasia Often it is difficult to decide upon the real nature of the tissue, i e chondro osseous area The arterioles and more especially the venulae give rise to vascular proliferations which begin with full vascular budding (Figs 5 and 6) This vascular hyperplasia is less intense than in the active stage of Paget's disease

In the non pressure area, which reveals a peculiar atrophy, the cartilage splits into two layers separated by spongy bone with fatty or mixed marrow The inferior layer which is embedded in the spongy bone shows the basophilic lines of the

distribution of mineral salts in Paget s disease, as judged from the microradiogram shows a great number of lowly mineralized areas which could account for Stanbury s high amount of exchangeable Ca The strange thing about all the diseases mentioned is that when you study them at the molecular level the relation between minerals and ground substance seems to be unaffected It is at the microscopic level that the organization seems to be disturbed

Black Prof Rutishauser have you done similar experiments with the bones that are osteoporotic after paralysis?

Rutishauser In the case of paralytic atrophy, which I examined by means of injection the atrophy was simple

Lacroix You have shown us a slide of osteoarthritis of the hip with a big hole filled with fibrous tissue near the articular cartilage It has been stated in this country that in such cases there is always a tunnel between the fibrous tissue and the articular space

Rutishauser Employing the serial section technique we found that this is not always the case

Fanconi Prof Rutishauser we know the Albers Schönberg disease with increase of bone structure is that also caused by an augmentation of vascularization?

Rutishauser I do not have enough personal experience with this subject

Nordin I think I am right in saying that the term Sudeck atrophy is used rather loosely in this country Prof Rutishauser I wonder if you would be kind enough to define exactly what you mean by Sudeck s disease ?

Rutishauser Sudeck's atrophy affects all the tissues (bone muscle skin) of a segment (generally an extremity) In the case of bone three stages are seen The second stage is an exaggeration of certain aspects in physiological healing of bone fractures The terminal phase is atrophic and completely different from physiological conditions

Nordin So it is really the atrophy that Lacroix showed us in X rays

Lacroix That would be what we would call the first stage

Nordin The question of the reversibility of the vascular changes is very fundamental to this argument and I did not quite see that they were reversible Did you have successive pieces of bone at the same site?

Rutishauser The question asked by Dr Nordin is very difficult to answer On the other hand the reversibility of the circulatory modifications in the remodelling of bone may be followed easily in Paget s disease My colleagues and I studied several cases of Paget s disease from their very beginning through their evolution to stabilization At this latter stage the medullary vascularization had returned to normal (Rutishauser E , Veyrat R and Rouiller Ch 1954 *loc cit*)

Follis Do you regard Paget s as primarily vascular?

Rutishauser The vascular transformations appear very early On the other hand dogs with artificial arteriovenous fistulae develop no histological picture which suggests any of the characteristic morphological elements seen in Paget s disease (Rutishauser and Veyrat 1954 Rutishauser Veyrat and Rouiller 1954 *loc cit*)

DISCUSSION

Stanbury We have certain data which may relate both to the problem of the vascularity of bone and to the problem of the interchange of Ca between the extracellular fluids and bone We have given ^{45}Ca to a series of patients and measured the specific activity of blood and urine curves with respect to time for up to 3 weeks Table I is a fragment of data

Table I (Stanbury)

Name		*Age*	*Sex*	*Diagnosis*	*9 Day Ca pool g*
R P		50	M	Coronary heart disease	9 9
Wa W		26	M	Friedreich s ataxia	17 2
F W	(1)	18	M	Hyperthyroid	245 7
	(2)	19		Euthyroid hypoparathyroid 1 year following thyroidectomy	84 6
L S	(1)	31	F	Hyperthyroid	68 1
	(2)	31		Early myxoedema 4 months following thyroidectomy	22 9
C L		38	F	Hyperthyroid	46 0
J B		26	M	Hyperthyroid	83 6
W W	(1)	42	F	Myxoedema	4 2
	(2)	42		Euthyroid 4 months on treatment	12 1
B M	(1)	39	F	Myxoedema	4 4
	(2)	39		Euthyroid 5 months on treatment	17 2
A W		67	F	Paget s disease of bone	102 1
O H		59	F	Paget s disease of bone	331 2
E N		41	F	Hypoparathyroid 4 years following thyroidectomy	3 5

which I would like to show We have calculated the 9 day ' pool ' of Ca by dividing the quantity of retained ^{45}Ca in the body by the specific activity at 9 days We studied two normal subjects At 9 days there was 9 9 and 17 2 g of exchangeable Ca In the patients with thyrotoxicosis you will notice the remarkably high figures Myxoedematous patients have a small pool of exchangeable Ca The myxoedematous patients when treated and restudied have a higher pool value

One of the things we speculated about was whether the observed phenomenon might represent vascularity of bone We studied two patients who had Paget s disease In these patients the 9 day Ca pool was very high being 103 1 and 332 9 g One of the things which these figures appear to mean is the remarkable exchangeability or dynamic state in which the bones exist and this seems to be particularly true in those patients who have thyrotoxicosis and Paget s disease

Follis Dr Stanbury have you any data on hyperparathyroidism?

Stanbury Not that I know of There was one patient to be studied after I left home These studies take a long time

Engstrom We have studied several of the bone diseases Paget s disease osteogenesis imperfecta marble bone disease and others The

SOME OBSERVATIONS ON EXPERIMENTAL BONE DISEASE

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THE normal sequences encountered in endochondral bone formation can be divided into three main categories (1) growth of cartilage, (2) maintenance of a fine balance between osteoblastic and osteolytic activities of the bone cells and (3) deposition of inorganic materials in cartilage matrix and/or bone matrix (osteoid) Derangements in each of these three categories may be observed in bone disease naturally occurring in the growing child or in the adult In the experimental animal one can produce at will changes which precisely duplicate many of the alterations seen in the human One can sometimes go even farther and create changes which have no counterpart in human disease

We should like to present some interesting, though diversified, examples of experimental bone disease which fit into these categories The changes which we wish to discuss will indicate a few of the large number of unsolved problems which concern the growth of normal cartilage and bone This presentation might better be entitled "A miscellany of unrelated and peculiar changes which may be produced experimentally in cartilage and bone and which may some day throw light on the biochemical activities of these structures"

When the dietary of an animal, or a human, too, for that matter, is restricted calorically or by the removal of one or more of any of the essential elements, amino acids vitamins or fatty acids, growth of cartilage stops, sometimes very abruptly The cells cease proliferating, in particular the zone of hypertrophic cells becomes narrower and narrower (Fig 1) Of course, the influence of the dietary restriction is

Lacroix Do you know of any possibility of reproducing experimentally something which might be like Paget s disease?

Rutishauser Bone grafts of Pagetic bone in full activity have never given the expected results in rhesus monkeys When transplanted to the temporal region of the cranium these grafts are always absorbed and often there is bony scar formation

Nordin Dr Follis is it a fact that vitamin A deficiency in rats gives a bone picture that is superficially not unlike Paget s disease?

Follis Grossly superficially

Nordin There is an increased turnover of bone, isn t there?

Follis I do not know what Prof Rutishauser may feel the Boston people think that the primary defect in Paget s is increased destruction and I think that with the exception of the vascular changes that is the first thing one sees The earliest changes in Paget s are really osteitis fibrosa

Rutishauser We may schematize the development of Paget s disease for a given area and divide its evolution into four phases phase IV corresponding to a stabilization of remodelling The morphology of the bone proper remains pathological

Follis I agree with you that if you look at enough sections you will find it going on but I think the destruction predominates 3 1, or in some fields 3 0

Rutishauser I do not think it is possible to make such oversimplifications



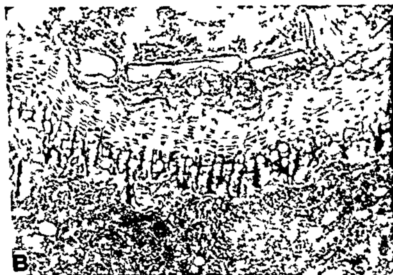


Fig 1 A Epiphyseal cartilage of normal rat to show width and orderly row formation of hypertrophic cells B Epiphyseal cartilage from rat on a protein deficient diet for 3 weeks (same magnification as A) to show great reduction in width of cartilage All types of cells are affected

also apparent on the activities of the osteoblast This is particularly true in ascorbic acid or copper deprivation Ordinarily, though, the effects on cartilage are often much more pronounced Certain simple histochemical procedures indicate, for instance, a decrease in glycogen and alkaline phosphatase activity (Follis, 1955*a*) As is well known, when the dietary becomes adequate again, growth of the cartilage *begins anew and there results an overproliferation of new bone* on the framework provided by excessively produced calcified matrix of the cartilage

The ease with which one can study alterations in the growth of cartilage by this means has prompted us to investigate the effects of purified growth hormone of the anterior lobe of the hypophysis* on the epiphyseal cartilage of rats which had completely stopped growing as a result of dietary restriction (Follis, 1955*a*) The problem, of course, was to find out if this disturbance of growth was primarily due to lack of growth hormone, since the change is identical with what is seen in the hypophysectomized animal It was found that when the hormone was administered in amounts more than necessary to promote growth in hypophysectomized rats, no effects could be elicited in the animals whose dietary had been restricted It is thus apparent that the missing calories or specific nutrients are necessary for the cartilage cells to reproduce and form matrix It will be recalled that from the very beginnings of the modern science of nutrition, growth and reproduction have been used over and over again as yard sticks for an optimal dietary

A second and particularly intriguing alteration in the growth of cartilage results when sweet pea seeds (*Lathrus odoratus*) are incorporated in the diet (Ponseti 1954) It has, of course, recently been shown that the active principle is β amino propionitrile (Bachhuber, Lalich and Angevine, 1955) When actively growing rats are placed on a diet containing 50 per cent ground sweet pea seeds† within 24 hours changes appear in

* Kindly supplied by Armour and Co Chicago Ill
† Kindly supplied by Ferry Morse Seed Co Detroit Mich

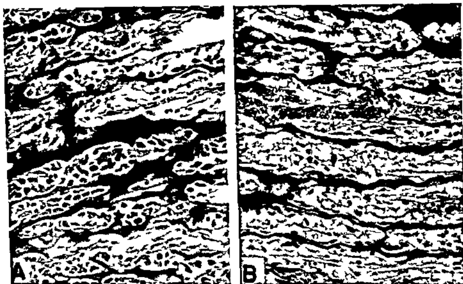


FIG 3 A Portion of metaphysis from control pig, to show spicules of calcified cartilage matrix upon which bone is being deposited Note large numbers of osteoblasts B Same region from a copper deficient pig to show thin spicules of matrix devoid of bone Note also the lack of cellularity





Fig 2 A Epiphyseal cartilage from rat which had been on a ground sweet pea seed diet for 48 hours Note clefts in cartilage matrix between cartilage cells B Epiphyseal cartilage after 8 days on the diet There is complete disorganization of the cartilage with large numbers of seemingly hypertrophic cells

the epiphyseal cartilage (Follis, 1955a) There is an increase in the zone of hypertrophic cells, and as time goes on a thick jumble of such cells is found (Fig 2) "Jumble" is used because the cells lose their orderly arrangement and form a thick band of large structures whose normal interrelationships are totally disorganized From the beginning, too, a change is found in the matrix of the less differentiated cells Clefts appear and the columns are spread apart As time goes on the adherence of the cells to one another seems to become less and less so that when one cuts through the epiphyseal cartilage with a sharp razor the normal resistance is completely lost The epiphyseal plate has become a sort of pulpy mass which appears in the fresh state, under the microscope, so lacking in cohesion that individual cells are found floating about by themselves We are at the present time studying certain histochemical aspects of this problem The change should interest certain members of this group who are investigating the deposition of ^{35}S in various areas, particularly cartilage

When one comes to take up the many alterations which may result from disturbances in the fine balance between bone formation and destruction there are any number of experimental situations which might be discussed We shall restrict our remarks on this subject to one the very interesting reaction of the osteoblast to a deficiency of copper Certain changes in the skeleton have been noted in animals grazing in areas whose soil is deficient in Cu Gross alterations designated as "osteomalacia" had been reported (Davis, 1950) We were much intrigued several years ago when Drs James Baxter and Judson Van Wyk brought us some bones from dogs which had been on a Cu deficient regimen (Baxter and Van Wyk, 1953) Clinically the disease was characterized by multiple spontaneous fractures with resultant deformities X ray studies revealed extreme rarefaction of the skeleton Grossly, at autopsy, the cortices of the long bones were extremely thin the epiphyses appeared wider than normal When the cortex was studied microscopically (Baxter, Van



FIG 4 Portion of cortex of tibia from rat which had received parenteral Sr (18 mg /100 g) each day for 8 days Note large amount of light staining material (osteoid) surrounding trabeculae of bone

Ca low diet stimulated growth in their rats More recently Shorr and Carter (1952) have used Sr as an adjuvant to Ca in the "remineralization" of the skeleton in various bone diseases in man

In some preliminary studies on rats, $SrCO_3$ was incorporated at a level of 4 per cent in a diet of adequate Ca and P content We were impressed by the relatively slight changes at the cartilage shaft junction though the amounts of osteoid in the shaft were prodigious This prompted us to administer various amounts of Sr intraperitoneally The osteoid continued to appear (Follis, 1955b) When the animals were starved and bone growth had ceased, parenteral Sr stimulated the formation of new osteoid (Fig 4) In healing fractures and in old animals Sr administration led to the appearance of excess osteoid. It thus became apparent that Sr seemed to stimulate new matrix formation This may have therapeutic implication and may explain some of the clinical results of Shorr and Carter (1952). The lack of calcification of the osteoid formed may be due to some inherent abnormality in it or to some interference by Sr in the calcification mechanism, perhaps by competing with the metal complex of the enzyme systems of cells concerned with calcification

Studies of certain chemical constituents of the serum of animals given parenteral Sr and showing excessive osteoid reveal no changes in Ca, P or alkaline phosphatase concentrations The effects of Sr are to be investigated in several directions in the near future

The second puzzling situation which we would like to mention is found in rats to which toxic amounts of vitamin D are given This state has for many years been known as "hypervitaminosis D rickets" (Ham and Lewis, 1934) and has been an enigma to a number of investigators including ourselves No changes are found in the epiphyseal cartilage, in fact, as a result of the toxicity there is cessation of growth of this tissue There is, however, new formation of osteoid which fails to calcify in the face of hypercalcaemia and hyperphosphataemia (Follis, 1955c) In the rat when levels of Ca and P are up to

Wyk and Follis, 1953), a marked decrease in thickness was found in the Cu deficient animals There was apparently a definite decrease in osteoblastic activity in the endosteal areas where destruction appeared to be proceeding in normal fashion At the cartilage shaft junctions of the Cu deficient animals the epiphyseal cartilages were increased in width as compared with controls The reason for this is not clear and need not detain us here In the metaphyseal regions an excess of calcified cartilage matrix upon which bone matrix was not being deposited was found Our interpretation of this was that the matrix formative functions of the osteoblast were impaired by Cu deficiency, while those of the cartilage cell were not This dissociation is frequently seen, for instance, in scurvy, osteogenesis imperfecta, etc

More recently (Follis *et al* , 1955) we have studied a series of Cu deficient swine Changes in the epiphyseal cartilage were not pronounced in the swine However, a prominent "lattice" of calcified cartilage matrix was present and was unsupported by bone (Fig 3) There was a definite reduction in osteoblastic activity This led to fractures quite reminiscent of scurvy, though unlike this disease there was no osteoblastic prolifera ation From these two series of observations in dogs and swine it would appear that Cu has a specific effect on osteo blastic activity Like ascorbic acid deficiency, a lack of Cu does not appear to affect chondrogenic activity

We should now like to turn to two puzzling situations in which there is excess production of bone matrix which does not readily calcify The first of these occurs when strontium is given to animals You are all familiar with Lehnerdt's (1910) observations on the appearance of a rickets like condi tion when excess Sr was administered to animals on a low cal cium diet This stimulated the Johns Hopkins group (Shipley *et al* , 1922) then working on rickets and vitamin D, to restudy Sr administration and to conclude that the changes observed by Lehnerdt had resulted from a low Ca, high P, diet and were not due *per se* to any action of Sr The Baltimore investigators (Shipley *et al* , 1922) did note that the inclusion of Sr in the

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DISCUSSION

de Bernard We did some experiments on the alteration of growth cartilage in thiamin deficiency, and found that the phosphatase content of this tissue was lowered

Rogers I was fascinated by your results with *Lathrus odoratus* Prof Follis Are they due entirely to the absence of glycogen?

Follis There must be something wrong with the matrix because the thing just goes to pieces Some of the disorganization that you see in Fig 2 is due to mechanical compression It is just a mass of pulp

Rogers It is not only disorganization of the arrangement of cells There is something wrong with the behaviour of the cartilage cells Is it possible that the β aminopropionitrile interferes with the formation of ground substance mucopolysaccharides? A failure to form polymerized chondroitin sulphate might well lead to tissue disorganization under stress such as you have described Have you tried anything other than β aminopropionitrile?

Meyer I have heard that it can also be done with aminoacetonitrile

Bélanger With Drs Comar Lotz and Visek of Oak Ridge we have just terminated a series of experiments on chronic fluorosis in pigs In this case we observed hypertrophy and also dissociation of the plate which are quite similar to that particular type of thing and also comparable to the lesion of vitamin D deficiency This condition is apparently related to a deficiency of the polysaccharides

Blaxter In cattle in the county of Caithness in the north of Scotland a condition of Cu deficiency associated with peatlands occurs in which there is a marked lightening of the black coat colour which is presumably an interference with melanin metabolism One thing which has not been studied up there is the fact that these animals develop very thin bones and always walk with a very characteristic stilted gait This same disease occurs also in certain parts of New Zealand I wonder whether Prof Follis has had any occasion to examine bones from animals which have been suffering from these so called defective bone conditions due to Cu deficiency in cattle or indeed the Cu deficiency syndromes which occur in sheep and which are associated with demyelination and locomotor ataxy

Follis This has been described in Florida also and has been called osteomalacia erroneously I think because the bones were not studied chemically and one should not use the term osteomalacia indiscriminately even amongst animals

16 mg per cent and 10 mg per cent, respectively, and inorganic material is being deposited in many areas myocardium, kidney, etc, this newly deposited organic matrix fails to show deposition of inorganic material in it! We have found that the serum of vitamin D poisoned rats with hypercalcaemia and hyperphosphataemia will readily calcify rachitic rat cartilage *in vitro* So, too, if the osteoid is incubated with normal rat serum, at Ca and P levels of say 10 0 and 9 0 mg per cent, respectively, after 24 hours there appears to have been a deposition of inorganic material in it when compared with controls We have detected this change histologically Obviously, a more precise method would be to determine if there were a difference in ash content Another peculiarity in hypervitaminosis D is the occurrence of alkalosis during a stage of toxicity Usually at death the whole blood pH is relatively normal, but several days before, we have encountered pH levels of 7 8 at 37° C with the Beckman blood pH electrode Another peculiarity is a fall in serum alkaline phosphatase activity For example, a decrease from a normal figure of 18 to a terminal value of 5 may be encountered This is of interest in view of the feeling that osteoblastic activity is going on at an accelerated rate

These then, are some examples of certain problems which have interested us in the past and which continue to intrigue us What little we have told you should further indicate how much there is to be learned about cartilage and bone

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Kodicek Reports have occasionally appeared in the literature that in hypervitaminosis, people have found overproduction not of osteoid but of bone

Follis I think the length of time that the animals live may be a matter of dosage This is the most extreme when the animals die in six days

Kodicek Could it be that there are two stages?

Follis Possibly and you can give an animal a course of vitamin D and not let him die The osteoid which I showed in Fig 4 has now calcified

Armstrong How would you explain the exostoses and the osteo sclerosis of chronic fluoride intoxication? Do we have to imagine that these are a result of overproduction of matrix?

Follis I would think so Of course a lot of this occurs at the sites of insertion of tendons and muscles and one looks upon that as perhaps being analogous to this in that an area of lowered resistance to stresses and strains with perhaps healing and overproduction of bone such as we mentioned might be the explanation of the changes Kodicek discussed in scurvy

Kodicek I take it that these hypervitaminotic animals were rats, you find about 60 per cent ash in normal rats and this seems rather high

Follis You can get it a little lower It depends on which bone—this was fat extracted bone

Kodicek Our results were on a fat free basis and we did not find lowering of the ash content in hypervitaminosis D

Follis Have you found a difference between hypervitaminosis D and cortisone?

Kodicek Not much the ash content was 41 and 43 per cent respectively I do not think the difference is significant Histologically your picture and ours agree

Dixon Can you reconcile the rickets producing action of Sr with Shorr s increased mineralization by Sr in human osteoporosis?

Follis Shorr gives fairly large quantities of Ca with the Sr and what we have to do now perhaps is to give Ca and not Sr and see whether the excessive amounts of Ca will deposit in matrix

Nicolaysen Sr has a dual effect it goes into the crystals and it interferes with metabolism at the cellular level

Follis A third effect perhaps may be interference with the calcification mechanism which has been demonstrated *in vitro* by Sobel

Nicolaysen This is a physicochemical concept

Fanconi I have seen many cases of hypervitaminosis D with osteo sclerosis of the metaphysis on the one hand and decalcification of the diaphysis on the other We always have hypercalcaemia in some cases a low or normal phosphate level in others a high phosphate level Very severe cases die of Ca poisoning

Follis These animals probably die of renal insufficiency, the kidney tubules become congested

Nordin The alkalosis is interesting What about K levels?

Howard I cannot tell you how it is produced but we get a high CO_2 and a low chloride in D intoxication just the same as one sees in K depletion We have studied one person during D poisoning for a week He was excreting more K in the urine than he was taking in in the diet but whether that was a typical week or whether K loss is the cause of the alkalosis I do not know It is most interesting

Nordin Dr Howard did you say there was uraemia there with a high phosphate?

Howard They had uraemia There is something in uraemic serum that prevents bone and cartilage calcification at much higher levels of Ca and P We could not find what it was it was not Mg or urea

seen because of the great variation in mineralization of different parts of the tissue

Among the bone disorders we have studied, there is one which shows certain typical morphological features not previously described (Engfeldt and Zetterström, 1954) The disease in question is termed hypophosphatasia in the literature The findings in this disease have such a character that they might be of general importance for the understanding of the process of mineralization We will give here a short description of the clinical and morphological findings in such a case This report concerns a 10 month old girl, the second child of healthy parents She weighed 4,200 g at birth She developed satisfactorily until the age of 3 months when anorexia and vomiting appeared Since birth she received 1000 units of vitamin D daily At the age of 5 months physical examination in a children's hospital revealed a generalized skeletal disease resembling rickets Signs of renal damage were found At the age of 6 months she was transferred to the Children's Clinic, Karolinska Sjukhuset On admission she weighed 5,380 g The fontanelle was bulging, there was a moderate rachitic rosary and swelling of ankles and wrists There were diastases of 2–3 cm between all bones of the calvarium The heart was of normal size The systolic blood pressure was 205 mm Hg There was no other clinical abnormality The child was hospitalized for four months until her death at the age of 10 months The condition was rather stationary during the first two months of her hospital stay, during two months prior to death she became progressively more ill, and the child expired with symptoms of pulmonary and cardiac failure

Laboratory investigations showed a slight normochromic anaemia The urine was generally acid A trace of protein was almost constantly present No microscopic haematuria, no casts, no abnormal excretion of amino acids or ketone bodies were found The excretion of calcium was somewhat higher than normal with a serum calcium concentration of 12 0 mg per 100 ml The blood chemistry is given in detail in

OSTEODYSMETAMORPHOSIS FOETALIS

A newly discovered characteristic skeletal disease showing low serum and tissue alkaline phosphatase activity (hypophosphatasia)

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A NUMBER of recently developed biophysical methods have been shown to be of great value in the study of mineralized tissues In our studies we are using X ray absorption and diffraction, micro interferometry and isotope labelling along with various histological techniques Using these methods, it has been possible to obtain certain quantitative data, and also to get new information concerning the properties of minera lized tissues Thus, in these investigations we have obtained quantitative information on the distribution of mineral salts, and the rate of rebuilding of bone could be estimated Further, the ultrastructure of the organic and inorganic components of mineralized tissue could be studied

Normal and abnormal bone and dental tissue have been studied in this way Thus, we have been working on bone disorders produced experimentally, such as hyperparathyroid ism, rickets and scurvy Various constitutional diseases of bone have also been examined, for instance Paget's disease, osteogenesis imperfecta, osteopetrosis and others No details of the results of these studies will be given here To illustrate what has been said we should like to present a microradiogram of a ground section of compact bone tissue in Paget's disease (Fig 1) In this microradiogram the difference in mineraliza- tion shows up as different shades of grey, thus light areas have a higher content of mineral salts than dark areas The characteristic mosaic pattern of the bone tissue is clearly

Table I There was a constant but mild hypercalcaemia The level of serum phosphate was on the whole normal Alkaline phosphatase activity of serum was determined according to Buch and Buch (1939), with this method the normal activity throughout infancy is 10–20 units In the present case the alkaline phosphatase activity of the serum was lower than normal.

In order to exclude a phosphatase inhibitor in the serum of the patient it was mixed with serum from another case with high serum phosphatase activity From Table II it is evident that there were no substances present that might have inhibited the phosphatase activity

Table II
ALKALINE SERUM PHOSPHATASE ACTIVITY

Experimental condition	*Tested immediately*	*Tested after 3 days storage*
	Units	*Units*
Patient	4	2
Control case	45	14
Patient + control case	24	9

The same volume of serum from the patient and from the control case (vitamin D refractory rickets) were mixed immediately following drawing of the blood samples The samples were stored in a refrigerator

There was a considerable increase of the α_2 globulin Such an increase is a common finding in various renal lesions

The patient reacted in a normal way to the subcutaneous injection of parathyroid hormone

X ray examination of the skeleton showed low mineralization throughout At all metaphyses considerable fraying fragmentation and deformation were seen There was a normal number of bone centres There was no evidence of renal calcification

The postmortem examination revealed a poorly developed and poorly nourished girl measuring 65 cm in body length

Table I

BLOOD CHEMISTRY

Date	*Serum Calcium*	*Serum Phosphate*	*Alk Phosphatase Activity*	*CO_2 Combining Power*	*Serum Chlorides*	*Total Bases*	*Non Protein Nitrogen*	*Total Protein*
1953	*mg per 100 ml*	*mg per 100 ml*	*Units*	*m equiv per 100 ml*	*m equiv per 100 ml*	*m equiv per 100 ml*	*mg per 100 ml*	*g per 100 ml*
10th March	11 0	4 8	3 3	—	—	—	—	—
2nd April	—	—	—	23	101	152	55	6 3
8th March	14 0	7 0	4 0	23	100	—	39	—
26th May	9 5	7 2	—	—	—	149	46	—
2nd June	12 2	—	4 0	—	—	—	56	5 8
25th June	12 9	7 3	4 0	—	—	—	—	—
10th August	—	—	—	13	—	—	59	6 0
20th August	—	—	4 0	—	—	—	53	—
24th August	12 0	6 6	—	—	—	—	—	—

Electrophoretic pattern of plasma proteins (16th May) showed increase of α_2 globulin (15 6 per cent against average normal value 5 8 per cent) at expense of albumin (53 5 per cent) The pattern was otherwise normal Total serum protein according to the method of Kjeldahl 7 69 g /100 ml Normal serum concentration of sodium and potassium Blood pH 7 4.

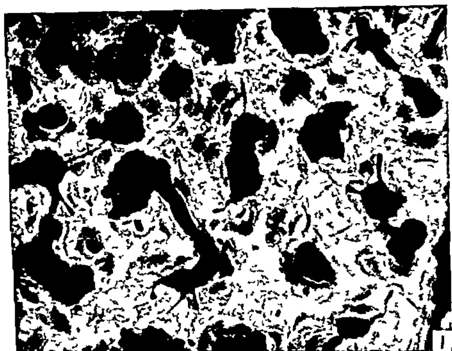


FIG 1 Microradiogram of ground transverse section of compact bone of femoral diaphysis from a case of Paget's disease

[facing page 262

The bones of the skull were soft and thin. The bones of the face were unremarkable. The thorax was small, and there were marked swellings of the costochondral junctions, especially of their interior aspect. The long bones were distinctly shorter than normal and the diaphyses were thin. Of the internal organs the kidneys attracted special interest. Their total weight was 54 g and the outer surface was irregularly granular. The parenchyma was firm and buff coloured. The cortex was moderately reduced in thickness. Microscopic examination of the kidneys showed marked interstitial fibrosis with quite abundant infiltration of inflammatory cells, mostly lymphocytes. Several shrunken and atrophic glomeruli with thickening, fibrosis and hyalinization of Bowman's capsules were observed. The tubules were atrophic. Frequently the convoluted tubules were widened and in several of them calcium casts were encountered. Interstitial deposits of calcium salts were demonstrated in addition.

Microscopic examination of the endocrine organs including the pituitary, thyroid, adrenals, ovaries, pancreas and all four parathyroids revealed normal findings.

Microscopic examination of the skeleton showed pronounced widening of the epiphyseal growth zones. The proliferating cartilage was irregularly calcified, the boundary towards the bone tissue being very irregular. In the metaphyses, subperiostally, there were abundant broad streaks of fibrous, partly hyalinized tissue with the appearance of osteoid. The morphological findings in the epiphyses were thus of the same type as those seen in severe rickets. The compact bone tissue in the diaphyses was of an immature lamellar type. Rebuilding of the compact bone tissue with formation of Haversian systems was only occasionally observed.

The bone tissue from this case was also subjected to the special examinations mentioned in the introduction. Concerning the technical data no account will be given here.

Microradiographic examination of the bone tissue from this case showed that its development did not correspond to the age of the subject. In this 10 month old child the diaphyses

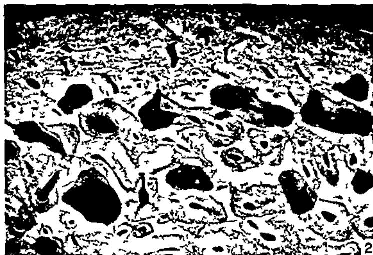


Fig 5 High magnification from an area of the same specimen as in Fig 4 The microradiogram shows resorption cavities and the formation of Haversian systems which appear however to be abnormal in many respects see text

Fig 2 Microradiogram of transverse ground section of femoral diaphysis (× 36 reduced 3/7) From a 10 month old infant with no signs of skeletal disease at death At this age there is a relatively broad outer circumferential lamella Most of the primitive bone is replaced by a bone tissue consisting of Haversian systems of different degrees of mineralization Owing to the high rebuilding intensity at this age a fairly large number of resorption cavities are observed

Fig 3 Microradiogram of transverse ground section of femoral diaphysis (× 36 reduced 3/7) From a newborn infant The bone tissue consists of primitive lamellar bone Note the broad highly calcified cementing lines arranged circularly to the bone axis In this period of development no distinct Haversian systems exist

Fig 4 Microradiogram of transverse ground section of femoral diaphysis (× 36 reduced 3/7) From the patient The bone tissue is in the main of the same type as in the newborn infant but poorer in cells A few resorption cavities may be seen


2


3


4

of the long bones by and large had the same appearance as is normally encountered at birth This is true of the structure as well as of the degree of mineralization (cf Figs 2-4) Fig 2 is a microradiogram of compact bone tissue of a 10 month old child without skeletal disease, Fig 3 is from a newborn baby and Fig 4 from the patient A divergent feature from what is observed in the microradiogram of the compact bone of a newborn baby is the absence of distinct cementing lines in the immature bone of our case Instead of the highly mineralized cementing lines found in normal bone the corresponding areas from this case are clearly visualized on the microradio graph because of a lower mineral content than that of the surrounding tissue The bone tissue of this case consists almost exclusively of primitive, quite uniform and highly mineralized bone Haversian systems and resorption cavities are found only occasionally The scanty, newly formed bone tissue also lacks the cementing lines that otherwise normally mark off old bone tissue from newly formed bone The recently calcified areas in the compact bone are also markedly less cellular than normal bone tissue of corresponding age (Fig 5)

In transmitted light, ground, decalcified sections of compact bone tissue examined in certain areas showed characteristic Haversian systems In polarized light the circular arrange ment of collagen bundles is typical for normal osteons The microradiograms obtained prior to the decalcification showed, however, that the systems had a low degree of mineralization and lack the normal gradient for calcium density Some Haversian systems were, indeed, entirely without mineral salts (cf Fig 6) Occasionally one finds systems in which only a sector of the system is mineralized These findings are never observed in normal bone tissue Nor does one find in cases of severe rickets Haversian systems with a complete collagen pattern but with a total absence of calcium salts

Examination of the ultrastructure of the bone tissue from this case has been performed using X ray diffraction techniques The mineral salts in this case showed the same

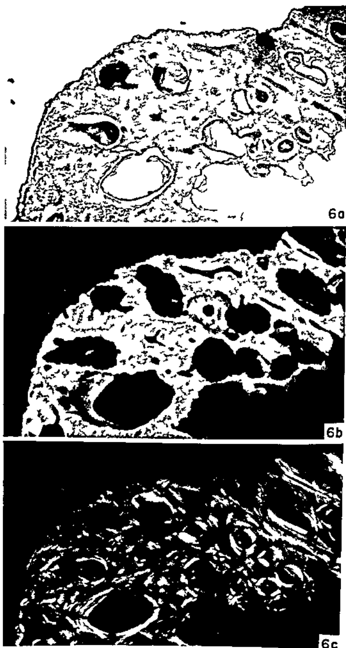


Fig 6 Transverse ground section of a specimen from the femoral diaphysis of the case described (× 30 reduced 2/5) a Decalcified section in transmitted light b Microradiogram of the same section before decalcification c The same section taken in polarized light after decalcification The Haversian systems formed have a normal collagen pattern Some of the systems are calcified others however are either completely uncalcified or mineralized only in a minor sector The collagen pattern in the primitive bone is irregular and the content of collagen is low

of a case which he termed "hypophosphatasia" In Rathbun's case, a male infant who died at the age of 9 weeks, there were metaphyseal changes resembling severe rickets In the blood serum there was a slight hypercalcaemia The alkaline phosphatase activity was extremely low in the serum, bone tissue, kidneys and in the intestines In 1953, Sobel and co workers described a case exhibiting some of the clinical features reported by Rathbun In a 19 month old girl severe rachitic changes and premature loss of deciduous teeth were observed There were no signs of renal disease The serum calcium level was normal and low serum alkaline phosphatase activity was found In the same communication preliminary incomplete data from five additional cases were given, all having roentgenological signs of severe rickets and low serum alkaline phosphatase activity At the meeting of the American Roentgen Ray Society in September 1954, Neuhauser and Currarino presented four similar cases which clinically, histologically and roentgenologically exhibited changes closely resembling rickets but which all showed an abnormally low alkaline phosphatase activity of the serum, bones and other tissues Diet and vitamin intake were normal These authors state that the skeletal disorder is characterized by defective bone formation and involves a few or all areas of endochondral or membranous bone formation while non-osseous growth processes continue It is of special interest that the parents of these children showed no osseous lesion but an abnormally low level of serum phosphatase in one or both

In the present investigation it has not been possible to establish any definite aetiology of the disease There are strong indications that the enzyme alkaline phosphatase in one way or another is concerned with bone formation It is still unknown, however, whether the enzyme acts upon the mineralization process or if its action is coupled to the formation of the organic matrix The low alkaline phosphatase activity of serum and certain tissues may either be a sign of a generally disturbed cellular function or the enzyme deficiency may be the primary cause of the disease

crystallographic pattern as does normal bone The alkaline phosphatase activity was determined in autopsy specimens from the skeleton and the kidneys, and the activity was found to be low both in the bone tissue and in the kidneys

Discussion

The most characteristic features of the skeletal disease described are inhibited skeletal development, considerably reduced rebuilding of bone and deficient mineralization of newly formed organic matrix The retarded growth of the extremities, the small diameter of the diaphyses and the wide diastases between the bones of the calvarium are signs of general inhibition of bone development

The cause of the renal lesion cannot be fully established It was present at the first examination, and at autopsy the kidney showed changes of nephrocalcinosis In infancy, this type of renal disease may develop as a result of different disease processes Chronic acidosis is one but the case presented did not reveal any signs of acidosis Primary hyperparathy roidism is another possible cause of renal calcification However, the parathyroids did not show signs of primary disease The persistent hypercalcaemia can hardly be the result of bone destruction, since the microradiographs showed bone resorption to be inhibited The renal calcifications were most likely the result of the hypercalcaemia This, in turn, was probably a consequence of an excessive resorption of calcium from the gastro intestinal tract excessive in propor tion to that deposited in the skeleton

Since the investigation did not reveal any evidence of the skeletal changes being secondary to renal damage, the skeletal abnormalities may be considered to be primary This conclusion is in harmony with the findings that the skeletal disease exhibited characteristics suggesting that it was of foetal origin

The case described here revealed clinical and pathological results resembling those of Rathbun (1948) in his investigation

The skeletal disease described shows on special examination of the bone tissue typical features allowing a differentiation from other diseases of bone, for instance rickets Therefore, we have suggested that it be termed osteodysmetamorphosis foetalis It seems reasonable to assume that the disease described is of foetal origin It may be considered to be genetically determined and to be caused by disturbed cellular function The disease can thus be referred to the same group of skeletal diseases as osteogenesis imperfecta and osteo petrosis The disorder appears to be a well delimited clinical and pathological entity which can be mild or severe It seems probable from the collection of cases reported recently that the condition may be more common than is generally assumed

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DISCUSSION

Dent Mr D C Cusworth and I have analysed the urine from six typical cases of hypophosphatasia in childhood * These all showed the same gross abnormality namely greatly increased output of phospho ethanolamine Paper chromatograms from the urine of one of these cases and from her father who is normal clinically but has a low phos phatase level are shown in Figs 1 and 2 respectively

* We are most grateful to Prof R A McCance for having sent to us for amino acid analysis the first urine from a case of hypophosphatasia

PLATE I

FIGS 1 and 2 (Dent) Paper chromatograms of urine from a case of hypo phosphatasia (Fig 1) and from her father (Fig 2)

The urine taken for each chromatogram contained 1 25 mg of total nitrogen It was placed on the cross at the right hand bottom corner and run first with phenol from right to left and then with lutidine in an upward direction The extra spot nearest the origin in Fig 1 is that due to phosphoethanolamine which is excreted in grossly abnormal quantities. The spot in the top left hand corner is due to an added marker The remaining spots in the central part of the chromatogram are the amino acids always readily detectable in normal urine namely serine glycine taurine alanine, glutamine histidine and methylhistidine

It is quite remarkable how the chromatograms from various affected cases from both sides of the Atlantic resemble each other (see also Fraser D, Yendt E R, and Christie F H E (1955) *Lancet* i 286) We are especially intrigued by the fact that the urine sent to us by Dr Henneman (Boston) from a patient who seems to be an adult case of hypophosphatasia, also shows an identical amino acid chromatogram Generally speaking the clinically normal relatives who have low phosphatase levels do not show an increased phosphoethanolamine output as marked as that shown in Fig 1 but we have reported among the relatives of one of Dr Schlesinger s cases an apparently normal brother with low phosphatase who shows the typical hypophosphatasia excretion pattern on the first specimen to be tested (Schlesinger, B Luder, J and Bodian M (1955) *Arch Dis Child* 30 265) We have not confirmed this unexpected finding on further specimens, so we think the first result was due to an error of collection

Another possible abnormality in these urines is that the total amino nitrogen excretion is a little less than normal both in the clinically affected cases whom we presume to be homozygous for the abnormal gene and in the unaffected relatives with low phosphatase levels whom we presume to be heterozygotes We originally suspected this from inspection of the paper chromatograms The quantity of urine analysed on the chromatograms shown in Figs 1 and 2 is that which contains 1 25 mg of total nitrogen which is five times the quantity we normally take At this overload we expect the spots from the urinary amino acids of normal people to be stronger than those shown in Figs 1 and 2 We have now confirmed this by amino nitrogen determinations

With regard to the identification of the phosphoethanolamine this is now almost certain although we have not yet succeeded in isolating and analysing a pure sample The paper chromatographic analysis shows identity with synthetic material so also does the paper electrophoretic separation which depends on entirely different principles and for which we have to thank Dr H Harris Also its behaviour on the ion exchange resins used for its isolation has been exactly that anticipated for pure phosphoethanolamine

We wonder what this means The most exciting possibility is that phosphoethanolamine is indeed the true substrate for bone alkaline phosphatase and that it is appearing in the urine because of insufficient enzyme in the manner one anticipates for the metabolic block from an inborn error of metabolism However we must not jump too quickly to such conclusions for there may be other more important phosphate esters also involved and these may also be accumulating in the body and being got rid of in various ways It may be that we have only detected the phosphoethanolamine because we happen to be doing amino acid chromatograms on a routine basis

There are two interesting clinical points to discuss The first is that the children are severely affected in the first years of life by the bone disease and that they get better spontaneously We think it is more than likely that the survivors will again present later in life with spontaneous fractures after the manner of the most interesting case which

their vitamin D sensitivity it is the fact that these people seem to start off with a low phosphatase Because they are D sensitive, they then may get hypercalcaemia Now consider a normal person who is intoxicated with a large dose of vitamin D, or equally well consider one of these people who are sensitive to vitamin D and who has been intoxicated with a small dose In either case he ends up with a high plasma Ca and low alkaline phosphatase Now if you do not take a history or know anything else the biochemistry is the same in hypophosphatasia and in D poisoning namely, high Ca low phosphatase In the latter case the vitamin D lowers the phosphatase in the other the low phosphatase comes first and makes him sensitive to vitamin D There is some connection there but the actual mechanism is obscure

Follis Then the low phosphatase in vitamin D poisoning is well recognized?

Dent I think it is well recognized It is mentioned in a paper by Fanconi and de Chastonay (1950, *Helv Paed Acta*, 5 5) on vitamin D intoxication three out of six cases had pathologically low phosphatases In idiopathic hypercalcaemia we frequently have low phosphatases this is one of the definite pieces of evidence for D intoxication in this syndrome (Bonham Carter Dent, Fowler and Harper (1955) *Arch Dis Child* 30 399)

Nassim Why do you think these are D sensitive?

Dent I do not really know None of them had been properly metabolized yet Prof Engstrom thinks it is over absorbing Ca We have got to explain a high Ca with normal parathyroids It is difficult to think of other explanations than those based on vitamin D action

Black I would like to show a few slides of a child with hypophosphatasia This case is the same as that mentioned by Dr Dent a full description of this child and of the slides I am showing has been published (Schlesinger B Luder, J and Bodian M (1955) *Arch Dis Child* 30 265)

The first slide demonstrates the bulging fontanelle due to a subdural haematoma The next two slides of X rays show the extremely deficient mineralization in the skull bones and the considerable widening of the suture lines the latter due partly to the subdural haematoma and partly to inadequate mineralization in the region of the sutures The X rays of the long bones show, in addition to the features previously mentioned by Dr Engfeldt (disordered ossification at the metaphyses with cupping) some periosteal new bone formation The last slide shows the changes in the blood chemistry during 16 months observation The main changes on admission were (1) a low alkaline phosphatase (9 0 King Armstrong units) (2) a raised serum Ca (3) a raised blood urea (70 mg per cent) (4) a mild transient hyperchloraemia with a low normal plasma bicarbonate During the period of observation the serum Ca gradually fell to normal in spite of treatment with large doses of vitamin D (up to 50 000 units daily) but began to rise again at the end of the period after a further short course of vitamin D in a dosage of 50 000 units daily During the whole period the blood urea was raised at all except four estimations The blood inorganic P was normal except for a

Dr Henneman kindly reported to me and has given me permission to quote here A similar case was reported by H B Macey (1940, *Proc Staff Meetings Mayo Clinic* 15 789)

We do not know why they should present twice like this with bone disease but we recall that this happens with certain other bone diseases such as various forms of refractory rickets of hereditary origin Another curious feature is that some of these children have hypercalcaemia and renal damage a situation strongly suggesting sensitivity to vitamin D Could it be that they lack the 'apo enzyme which in normal people binds the vitamin D as prosthetic group and thus keeps it out of mischief? Whatever may be the meaning of all this it is interesting that we now seem to have another disease which can be diagnosed by paper chromatography

Armstrong Dr Dixon and I were wondering whether phosphoethanolamine is hydrolysed by phosphatase and, if so what the rate of hydrolysis is

Dent I do not know the rate It is hydrolysed quite readily by alkaline phosphatase Dr Bourne is already working on it from a histochemical aspect using phosphoethanolamine as a substrate for the alkaline phosphatase staining

Black A bone biopsy was done on the case which Dr Dent just mentioned the phosphatase was reduced as shown by histochemical methods and also by quantitative methods (Bessey and Lowry technique) The number of osteoblasts and osteoclasts is also thought to be reduced

Perkins Was the phosphatase lowered in other organs as well and if so in which organs?

Dent Yes in the intestine kidney, liver and bone not so much in other organs

Nassim When you say the children get better Dr Dent do you mean that if they survive they have normal development? Do they grow?

Dent They are radiologically better and they grow This adult which Dr Henneman told us about is now 5 1" tall which is reasonable

Nassim Does the alkaline phosphatase increase?

Dent No It is only the clinical medicine that is really difficult to understand in these hereditary diseases

Nordin Have you looked for this in idiopathic hypercalcaemia?

Dent No

Black There is no correlation between the presence of the spot and the low phosphatase in what you might call carriers is there? In some of the cases in the literature the parents or siblings have been described as having abnormally low phosphatase

Dent No there is not We still think there might be a very slight increase in the carrier but certainly nothing like these chromatograms from cases with the disease That first one I showed was in fact a carrier

Black So this child s brother has a normal urine and a low phosphatase?

Dent Yes he is a carrier His brother is healthy I am most grateful to Prof McCance for pointing out to me a very strange thing concerning

Dent Jonxis has sent us one or two urines from cases of scurvy and has analysed many more himself, and we have not noticed it I should think it is not present in scurvy You do get this spot in other diseases for instance you get it as a temporary phenomenon in liver disease It is the constancy of this thing in hypophosphatasia that is so interesting This should link it very closely with the abnormal gene action, especially since we have now also found it in an adult

short period during the first course of treatment on vitamin D when it rose for a short time to 6 mg per cent The alkaline phosphatase re mained at a very low level throughout, never rising above 9 units and sometimes dropping to 1 unit

It is a bit puzzling that the Ca appears to come down I do not know how we can explain that In justice to Dr Dent I think the Ca went up later There were toxic symptoms of hypercalcaemia and it had to be stopped One other interesting point is this development of craniosten osis in a very large number of these patients, not all It was so severe that this child had to have surgical interference in order to prevent the onset of optic atrophy

Nordin What was the evidence that the alkaline phosphatase was low before the Ca was high, Dr Dent? You gave this very nice contrast that in the hypercalcaemia you had a high Ca, perhaps due to D sensitivity, causing a low phosphatase and then you said it can be the other way round you can have a low phosphatase state and it leads to a hypercalcaemia Have you actually seen the low phosphatase before the hypercalcaemia?

Dent No I don't think so

Nordin So they might really be the same thing?

Dent All you can say about the hypercalcaemia is that it is incon stant, whereas the low phosphatase is much more constant Some cases do not have hypercalcaemia Hypercalcaemia seems to be an accidental thing, thrown in for good measure It does not seem to be actually necessary likewise the renal damage The child discussed by Dr Black is much better now clinically

Nordin The two conditions are very similar apart from your phos phoethanolamine, is that right?

Dent Yes

Nassim Do the idiopathic hypercalcaemic children show these very florid rickety changes?

Dent No, they show overcalcification

Nordin That is a very good point

Armstrong Only a part of the serum phosphatase is derived from the skeleton is that right?

Black I imagine so

Armstrong Is it possible then, that the very low amounts of phospha tase which you find in the serum are derived from say the liver and not at all from the skeleton?

Black If you analyse all the organs involved as has already been mentioned, they are all low There is some activity

Dent I think Prof Armstrong was suggesting that there might be more than one phosphatase and if we could only analyse for the real phosphatase from bone we might find it completely absent in the serum and in the organs

Perkins It would be rather interesting to know whether in a condi tion like scurvy, where you get low phosphatase in the bones and in the serum but not in the other organs, you get phosphoethanolamine in the urine

Dent Jonxis has sent us one or two urines from cases of scurvy and has analysed many more himself and we have not noticed it I should think it is not present in scurvy You do get this spot in other diseases, for instance you get it as a temporary phenomenon in liver disease It is the constancy of this thing in hypophosphatasia that is so interesting This should link it very closely with the abnormal gene action, especially since we have now also found it in an adult.

BONE AS A CRITICAL ORGAN FOR THE DEPOSITION OF RADIOACTIVE MATERIALS*

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THE range of the topic that has been suggested for this paper is so great and the literature pertinent to the subject has become so voluminous in recent years that, in a short communication of this type, a few cardinal examples must suffice to illustrate the thesis that bone is in fact a critical organ for the deposition of certain normally occurring radioactive elements and of others that have been rendered radioactive by artificial means The term 'critical" as used in this paper refers to the preferential deposition and retention of any radioactive substance in bone and especially to the fact that such retention may constitute a hazard by virtue of the emission of radiant energy into the tissue in which it is deposited and the subsequent production of undesirable and serious morbid processes More specifically, I should like to describe briefly the general character of the skeletal abnormalities produced by radium and mesothorium in man, abnormalities that, with minor variations, are characteristically produced by bone seeking radioelements and that can be readily reproduced in the experimental animal Although much has been written on radium poisoning in man during the past thirty years, it is surprising to discover that very little has been said about the histopathology of the skeletal lesions in this disease, which are generally and somewhat vaguely being referred to as "radiation osteitis"

Knowledge of the harmful effects of bone seeking radioactive materials has accumulated during the last three de

* Work performed under the auspices of the Atomic Energy Commission

cades, ever since Blum in 1924, Hoffman in 1925, and especially Martland in 1925 and in subsequent classic publications, called attention to the occurrence of a peculiar kind of bone disease in the celebrated dial painters of New Jersey These dial painters, it will be recalled, used a self luminous paint containing zinc pyrosulphate and small amounts of radium, mesothorium and radiothorium In the early years of this industrial process many dial painters succumbed to a progressive and often fatal illness, resulting from the ingestion, over periods of months and years, of considerable amounts of this paint through the notorious practice of tipping the brushes with their lips The more fulminating type of illness of some of the dial painters was characterized by rarefaction, necrosis and fractures of bone, combined with severe damage to the bone marrow which resulted in profound anaemia, leucopenia and agranulocytosis, the patients usually died from overwhelming infections There have been relatively few instances of this comparatively acute or subacute type of illness, which was caused by the ingestion and deposition of relatively large quantities of radioactive materials in the skeleton The more common picture of radium poisoning is that of an insidious and chronic disease, the symptoms of which as a rule do not appear for many years following exposure These chronic cases are the result of the fixation in the skeleton of relatively small quantities of radioactive materials The clinical course of patients with chronic radium poisoning is characterized by slowly progressive skeletal lesions appearing first as discrete and later as confluent areas of bone destruction and rarefaction, these in the fully developed cases give to the roentgenograms their characteristic mottled appearance These skeletal abnormalities may or may not be associated with abnormalities in the peripheral blood It is a distinctive feature of chronic radium poisoning that malignancies are likely to develop in the damaged skeleton and many instances of such occurrence have been recorded in the literature

Further evidence of the delayed toxic effects of radium salts has come from observations of patients who, during the

second and third decades of this century, had been treated with such compounds for a great variety of illnesses Over enthusiastic but misguided therapy of this type had become a well established procedure shortly after the discovery of radium, as pointed out by Looney and associates (1955) Skeletal lesions and blood dyscrasias identical with those of the dial painters have been observed in many of these patients Thus radium poisoning, although a rare disease, is now a well recognized clinical and pathological entity The clinical syn drome of the disease has been described recently in great detail in two exhaustive studies by Aub and associates (1952) and by Looney and his associates (1955) In each of these papers there are examples of dial painters as well as of patients suffering from "therapeutic" radium poisoning These papers should be consulted for study of the pertinent details of the clinical course of the disease as well as for an appraisal of the numerous physical, chemical and metabolic aspects of the aetiologic agents

This sketchy outline of radium poisoning has been given as an introduction to the description and illustration of a few pertinent histopathological observations on the bones of a radium dial painter The tissues, which have been made available to me through the kindness of Drs R J Hasterlik and C H Hatcher of the University of Chicago, were obtained from patient 313 in the series of cases recently reported by Looney and associates (1955) A more detailed description of the pathological anatomy of the bones of this and other patients with chronic radium poisoning is in preparation

Clinical history This patient had been a dial painter for 28 years when she died in 1954 at the age of 48 with a local recur rence of a fibrosarcoma of the right ischium She had begun work as a dial painter in 1924 at the age of 18 For the first two years of her employment she was in the habit of tipping the brush with her lips She had been in good health until 1948, when in two separate falls she twice fractured the shaft of the left femur within four months However, both fractures healed satisfactorily and the patient was able to return to

work In 1951, in the course of a routine examination, a roentgenographic survey of the skeleton was made, and skeletal lesions typical of radium poisoning were found in the skull and in several long bones There were no lesions in the pelvic bones Haematological examination showed no ab normalities In 1953 a carcinoma of the cervix uteri was diagnosed This was treated with radium and X-rays and a total hysterectomy was performed shortly thereafter There was no further evidence of this disease However, a month or two later a roentgenogram of the pelvis showed a large osteolytic lesion in the right ischial tuberosity and in the pubic bone This was treated with 2000 rep of X rays on the basis of a presumptive diagnosis of carcinoma of the cervix uteri, secondary in bone However, the lesion persisted and the patient was transferred to the University of Chicago Clinics for study and further treatment Biopsy of the tumour mass revealed a well differentiated fibrosarcoma arising in bone Local resection of the tumour was impossible and a right hemipelvectomy was therefore performed by Dr C H Hatcher in November of 1953 The specimen obtained at operation showed a tumour 11 cm × 15 cm in size which completely replaced the ischium and protruded into the pelvis, it also invaded parts of the acetabular fossa The patient recovered from the operation but re entered the hospital several months later with a local recurrence of tumour at the site of operation She died eight months after the operation The total body radium burden was 1 3 μc at death

Histopathology As was to be expected from the appearance of the roentgenograms, the lesions in the skeleton were found on histological examination to be distributed widely throughout many of the bones that were available for study There were considerable differences in degree of bone involvement not only from bone to bone, but also within different parts of the same bone Severe abnormalities were seen in the bone tissue itself as well as in the bone marrow, in the former they involved dense cortical bone as well as the spongiosa Lesions in the bone marrow were focal in character and generally restricted

to the portions of the bone marrow immediately adjacent to the trabeculae, or to bone surfaces in the diaphyses and elsewhere

The cortex of the shafts of the long bones varied considerably in thickness, in fact there were some areas without any cortical bone at all, as in the metaphysis of one tibia Numerous small cavities or holes were distributed irregularly throughout the cortex, they measured from fractions of a mm to as much as six mm in a longitudinal section This fenestration or porosity of the bone was a very characteristic feature (Fig 2) Some of the smaller defects undoubtedly represented old resorption cavities with failure of new bone to be laid down on them All of the defects, small and large, were now filled either with fat tissue or with a mixture of fat cells and a relatively acellular and often oedematous connective tissue in which a few small vascular channels were found on occasion Active resorption cavities containing a number of osteoclasts but relatively few osteoblasts, were seen here and there in all of the bones

A second and equally striking feature of the skeletal pathology consisted of a highly atypical and peculiar osseous tissue that was found deposited in many parts of the cortical bone This differed from the surrounding normal bone not only in its distinct basophilic staining qualities of varying intensity, but also and especially in the highly irregular manner in which it was deposited Occasionally, a concentric layer of such atypical osseous tissue was seen to alternate with a layer of more normal appearing bone in a single Haversian system (Fig 3) Extensions of this atypical material were often seen as small plugs in the lumina of Volkmann canals, a similarly *abnormal type of bone was also observed in parts of* the periosteum

It was difficult to ascertain the degree of viability or non viability of these bones Careful examination has shown, however, that no major portions of the bone tissue were completely devitalized i e clearly necrotic On the other hand, many empty lacunae were noted in parts of the dense cortical bone

Fig 1 Cross section of normal human tibia (Reproduced from Petersen 1930) (× 21)

Fig 2 Cross section of femur from patient with radium poisoning showing fenestration of cortex foci of atypical bone and altered architecture (× 20)

[facing page 26

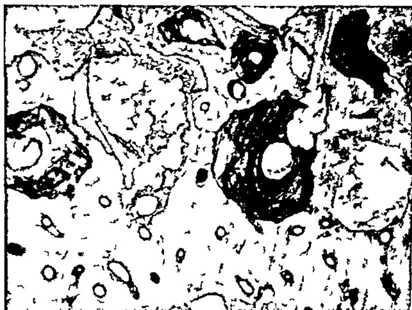


FIG 3 Atypical Haversian type of bone from femur showing irregular pattern of deposition (× 40)

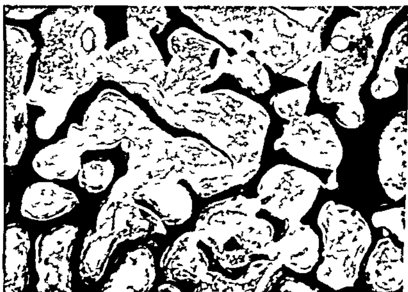


FIG 4 Extensive peritrabecular fibrosis in ischium (× 15)

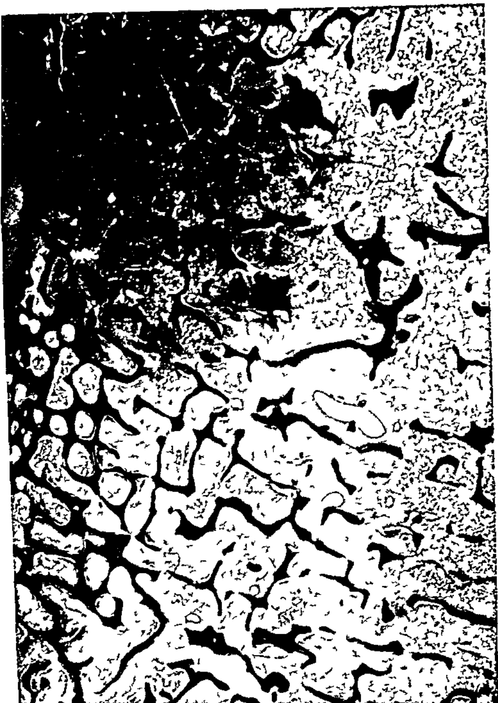


FIG 5 Small portion of fibrosarcoma of ischium infiltrating bone and bone marrow Fibrosis and oedema of marrow (× 8)



FIG 6 Vertebra of rat with plutonium poisoning showing two circumscribed areas of fibrosis around damaged bone and an osteogenic sarcoma arising from one of them (× 18)

and cell ghosts were seen in others Lacunae with and without osteocytes were apparently distributed at random, although one had the impression that there were fewer intact osteocytes in those parts of the bone that showed other evidence of injury

A third, and perhaps the most significant component of the skeletal damage consisted of a very conspicuous fibrosis involving bone and bone marrow This was characteristically localized around bone trabeculae and along endosteal surfaces of cortical bone (Fig 4) In the most severely affected bones, as in the femur and the ischium, large numbers of trabeculae were completely surrounded by several layers of dense and often hyalinized connective tissue, whereas in other parts of the skeleton the fibrosis consisted only of thin patches of a more cellular connective tissue covering not more than small fractions of the surface of otherwise intact bone trabeculae Curiously enough, there was no correlation between the extent of fibrosis around a given bone trabecula and damage in the osseous tissue of the trabecula itself In fact, most of the trabeculae showed intact and viable bone tissue The old, hyalinized foci of fibrosis frequently showed calcification and metaplastic bone formation This had led to an accumulation of heterotopic osseous tissue, often of imperfect quality, between and in apposition to bone trabeculae and on the endosteal surfaces of the cortex In the latter location such heterotopic bone was often intimately interwoven with pre-existing cortical bone

The tumour mass in the ischium was a well differentiated, very cellular fibrosarcoma (Fig 5) composed of interlacing bundles of very small cells showing a fair degree of pleomorphism of nuclei and cytoplasm and very little intercellular substance There was no bone formation It is likely that the tumour originated from cells of the old peritrabecular fibrous tissue that was so abundant in the ischium

Thus three principal morbid processes dominated the picture of the skeleton of this patient after many years of exposure to radiations from radium and mesothorium (and their decay products), fixed in the bone tissue firstly, a

widespread osteoporosis of a peculiar type, secondly, the deposition of atypical osseous tissue which was formed (a) in the course of the normal processes of reconstruction of bone of the Haversian type and (b) as a consequence of metaplastic and heterotopic bone formation in fibrous tissue, and thirdly, a fibrosis of the bone marrow, characteristically restricted to the endosteal surfaces of trabecular and cortical bone but not necessarily related to bone damage *per se* This reactive fibrosis of long standing has been considered to be of special significance, since it is thought to be the source of the malignant tumour that eventually developed in the ischium of this patient

It is clearly impossible to reconstruct from a few specimens *of one patient the pathogenesis of so complex a lesion as has* just been described, a lesion which in the final analysis is the end product of a long series of acting and reacting forces of injury and repair which have occurred continuously for 28 years, as in the present instance These lesions obviously are the result of damage to all component parts of skeletal tissue, including osteocytes and osteoblasts, the vascular system and the organic matrix of bone, not to mention bone mineral itself It would be presumptuous to try to single out damage to any one of these numerous components as being primarily responsible for the total picture, although it is probable that there are individual differences in susceptibility to damage by ionizing radiations among cells in this system, just as there are in other systems Unfortunately, it has not been possible to correlate the pathology as seen in this patient, with the distribution of radioactivity in various parts of the skeleton The elucidation of the relationship of hot spots (Aub *et al*, 1952, Looney and Woodruff 1953) to the degree of damage must therefore be left for further studies

Discussion

A large body of evidence has been brought forward in recent years to show that other foreign elements in addition to

radium and mesothorium are deposited and fixed in the skeleton Of special interest in this connection are (1) the heavy elements uranium, plutonium and other transuranic elements of the actinide series, and (2) the radioactive isotopes of a number of elements in the middle of the periodic table which are formed in abundance in the fission of uranium or plutonium The metabolism of transuranic elements and of fission products has been a matter of intense study in recent years, and the subject has been reviewed by Hamilton in several publications (1948, 1949) There are fourteen fission products which represent almost all of the radioactivity of a fission product mixture when it has been allowed to decay for a week, it is apparent from the experimental work of Hamilton and of others that bone is the principal organ of retention for nine of these The half lives of eight of these elements range from twelve days to twenty five years Bone is also the principal organ for the deposition of plutonium and a number of other elements of the actinide series The outstanding metabolic characteristics of the fourteen fission products and of some members of the actinide series are summarized in Table I which has been reproduced from one of the papers by Hamilton (1948) Physiological and physicochemical factors governing the incorporation and pattern of distribution of radioelements in bone have been discussed in detail by Neuman and Neuman (1953) and by McLean and Urist (1955) A few of the essential points that have emerged from the numerous studies on the metabolism and retention of radioactive substances in bone may be summarized as follows

1 Elements which have an affinity for the skeleton may be divided into two categories, namely those like calcium, strontium and radium which are known to be associated with the mineral phase of bone tissue, and those like yttrium, plutonium and the rare earths which have a different pattern of deposition

2 Bone growth and reconstruction largely determine the pattern of deposition of calcium and phosphorus and they also govern the pattern of deposition of foreign elements such as

Table I

SUMMARY OF THE METABOLISM OF THE PRINCIPAL MEMBERS OF THE LONG LIVED FISSION PRODUCTS AND CERTAIN OF THE FISSIONABLE ELEMENTS IN THE RAT FOLLOWING PARENTERAL AND ORAL ADMINISTRATION (HAMILTON 1948)

Radio element	*Half life*	*Fission yield per cent*	*Per cent oral absorption*	*Per cent accumulation in principal organ of retention*	*Rate of elimination from principal organs of retention*	
^{89}Sr	53 d	4 6	5–60	70 bone	bone	>200 d
^{90}Sr	25 yr					
^{140}Ba	12 8 d	6 1	5–60	60 bone	bone	>50 d
^{131}I	8 0 d	2 8	100	20 thyroid	thyroid	>30 d
^{135}Cs	33 yr		100	45 muscle	muscle	15 d
^{91}Y	57 d	5 9	<0 05	65 bone	bone	>500 d
^{140}La	40 hr	6 1	<0 05	70 liver 30 bone	liver bone	10 d >25 d
^{141}Ce	28 d	5 7	<0 05	50 liver	liver	10 d
^{144}Ce	275 d	5 3	<0 05	25 bone	bone	>100 d
^{143}Pr	13 8 d	5 4	<0 5	35 liver 50 bone	liver bone	10-d >100 d
^{147}61	3 7 yr	2 6	<0 05	55 liver 35 bone	liver bone	10 d >100 d
^{95}Zr	65 d	6 4	<0 05	35 bone	bone	>100 d
^{95}Cb	3[illegible]-d	6 4	<0 5	30 bone 25 blood	bone blood	30 d 1 d
^{103}Ru	42 d	3 7	<0 05	3 5 kidney	kidney	20 d
^{106}Ru	1 yr	0 5	<0 05			
^{127}Te	90 d	0 033	25	15 blood	blood	15 d
^{129}Te	32 d	0 19	25	6 kidney	kidney	15 d
^{133}Xe	5 3 d	4 5	*Distribution proportional to fat content of body half time in the body two hours*			
^{227}Ac	13 5 yr		<0 05	50 liver 30 bone	liver bone	>4 d >4 d
^{231}Th	24 5 d		<0 05	50 bone	bone	>200 d
^{231}Pa	3×10^4 yr		<0 05	40 bone	bone	>100 d
^{233}U	1 6×10^5 yr		<0 05	45 kidney 20 bone	kidney bone	5 d 60 d
^{239}Np	2 2-d		<0 05	65 bone	bone	>50 d
^{239}Pu	2 2×10^4 yr		0 007	75 bone	bone	>2 yr
^{241}Am	500 yr		<0 05	60 liver 25 bone	liver bone	10-d >1 yr
^{242}Cm	150-d		<0 05	60 liver 25 bone	liver bone	10 d >1 yr

strontium and radium In young growing animals as well as in adults, discrete areas of high radioactivity (hot spots) can be correlated with areas of bone growth and reconstruction (Amprino, 1951, Towsey, Owen and Vaughan, 1953, Engfeldt *et al*, 1954) Age and mineral metabolism have little if any influence on the amount and manner of deposition of radioactive elements such as plutonium and cerium (Copp, Axelrod and Hamilton, 1947), thus indicating a marked difference in metabolism and in manner of deposition, a difference that had already been noted from earlier autoradiographic studies

3 Once incorporated in bone, and regardless of the manner of initial deposition, most radioactive materials remain there for long periods of time The type of damage to bone and bone marrow that is produced in experimental animals by the administration of bone seeking isotopes closely resembles the lesions that have been described in the first part of this paper In the case of tumour formation, this is illustrated in Fig 6, which shows a longitudinal section of a vertebra of a rat with plutonium poisoning The essential feature of the lesion consists of two densely fibrotic scars surrounding a number of damaged bone spicules These two scars are characteristically located in the distal portion of the trabecular bone at either end of the vertebra (metaphyses) An osteogenic sarcoma which arose from one of the foci of fibrosis now completely fills the space between the old scar and the epiphyseal plate The bone that is formed by the tumour cells is seen as irregular spicules that appear black in the photomicrograph

The early sequences of injury and repair in bone following the injection of representative bone seekers have been described in great detail by Heller (1948) Tumour formation in bone following the administration of plutonium and of various fission products, notably strontium has been reported previously from this laboratory (Lisco Finkel and Brues, 1947 Brues 1949) These problems continue to receive attention in efforts to express the toxicity of radioactive materials in terms of efficacy of tumour production in relation to dose (Finkel, 1953) and in studies of the pathogenesis of neoplasms

of the skeleton under these experimental conditions, such studies may eventually permit one to draw conclusions concerning certain mechanisms of carcinogenesis in general

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DISCUSSION

Rutishauser We examined 42 femur necks which had belonged to people under 70 years of age Calcifications, identical with those found in Dr Lisco s slides are relatively frequent at that age (Dufour J J (1953) *Helv chir Acta* 19, 1 *Rev Chir Orthop* Suppl 1 p 41)

Lisco This is of great interest to me particularly in view of the fact that radiation injury is said to increase ageing processes

Dent May I inquire if the X rays like the one that you showed of the tibia are like that all over the body?

Lisco They are like that in the tibia primarily and in the small bones of the foot, in the femur and in the humerus There was much less in the spine

Nassim In the doses you have used was there any incidence of blood diseases in the experimental animals you showed which had tumours getting on for 600–900 days?

Lisco No there was not, and there were very few spectacular lesions in the bone marrow

Nassim Radiologists are much more prone to get leukaemia aren t they?

Lisco Yes they are but that is X ray and gamma irradiation

Black Is there any reasonable correlation between the latent period for tumour production and the lifespan of the animal involved, or are the dosages too difficult to control?

Lisco Yes there is a correlation between latent period and lifespan of the animal However I am not sure exactly what fraction of the lifespan this is in each species

Meyer Do you observe the simultaneous appearance of various histologically distinct types of sarcoma at the same time in your experimental animals?

Lisco In an animal developing more than one tumour the tumours are as a rule of the same appearance

of the skeleton under these experimental conditions, such studies may eventually permit one to draw conclusions concerning certain mechanisms of carcinogenesis in general

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off all food, and the differences in the Ca and P behaviour in the two or three days during that starvation were very dramatic In those on D, there was practically no fall in serum Ca, and those which were D-deficient and had had exactly the same diet, had a very marked fall in serum Ca There was, of course, considerable difference in their bones I do not know whether or not that is an explanation for differences in hyperparathyroidism, but I suspect that it is some environmental factor and as far as diet is concerned we cannot deduce what this is from historical evidence It seems to me that everybody gets a more or less adequate amount of vitamin D in adult life.

Dent Do you accept that there is a division into two forms of hyperparathyroidism and that you can nearly always say to which of the two a person belongs?

Howard There are holes in the bones of some and not of others but I do not see that that makes one have a different disease from the other

Dent What about the differences in phosphatase level?

Howard Rarefaction and high phosphatase do not always run parallel Nor does the individual who has a high phosphatase in the blood when you remove the tumour always have a hypocalcaemia after the tumour is removed, nor does his phosphorus necessarily stay down longer after the tumour is removed than in the other case That is to me a very fascinating phenomenon At least 50 per cent of our cases stay down for months, i e the serum P remains low after the Ca came down to normal very little Ca is coming out in the urine everything seems to be perfectly all right the serum P may remain low for as long as a year But this low serum P after operation does not parallel the level of the phosphatase or the presence or absence of radiographic evidence of osteitis

Rutishauser The parathyroid cells belong to the water clear cell series of Getzowa whether they are small or large cells (Wernly, M (1946) *Helv Med Acta*, 13 Suppl 19)

Lacroix Since the main difference rests on the reactions of the skeleton, it may be that the skeleton itself is different in two subjects After all we do not know the proportion of young bone compared to old bone in two subjects of the same size and age

Dent That is my own preferred view at the moment that there are two different kinds of people or of skeletons How are we going to prove that? The trouble is that as Prof Rutishauser says there is no histological difference between the parathyroid glands in the ones with gross bone changes and in those who have no bone changes

Rutishauser The hyalin changes discovered by Crooke are important for bringing under one syndromal heading the primary

GENERAL DISCUSSION

Dent I suggest we go back to the discussion on the parathyroid hormone and its actions I would like to ask Dr Howard a question that always worries me and that clinicians and physiologists and everybody else do not do enough about Dr Albright made a very brave attempt to explain the reason for there being two kinds of hyperparathyroidism that with bone disease and a raised alkaline phosphatase, and that which is much more common with normal bone pathology, normal phosphatase and chiefly urinary renal symptoms As far as I know there is nothing in the pathology of the parathyroid gland to distinguish between the two Certainly bio chemically apart from the phosphatase differences there is nothing between the two that I know of The plasma levels the Ca balance changes are the same It seems extraordinary to me that people with hyperparathyroidism may all be apparently actively decalcifying as far as you can see from their balance data—of course you can only do this on a short term basis—but that only some of them should have radiological signs and great bone rarefaction while the others seem to have normal bones This to my mind, is the best evidence there is suggesting that there is more than one parathyroid hormone

Armstrong I thought Albright's explanation of the cases of hyper parathyroidism without skeletal decalcification is that these are persons who are heavy milk drinkers

Dent That is so and I am completely unconvinced by the Albright explanation that it is dependent on the Ca intake that if you had a high Ca intake it stopped you getting the bone disease We have taken histories and people with the bone form have drunk lots of milk and people with the other form did not like milk and so on

Howard Going back to our case histories we too could find no dietary distinction between those with bone disease and those with out This would not lead me to deduce that there is more than one hormone, but that there are two different types of persons with hyperparathyroidism I have no real explanation for it but persons do differ enormously I do not know what those factors are, but I do not believe that you need to bring in the parathyroid hormone as being different in one sort from the other In Dr Park's experiments —that I think were never published—on animals to which he gave rachitogenic diets (high Ca low P) one group was given vitamin D in adequate amounts and the other not He then took both groups

histological level only, being at once fibrous and osteomalatic Histologically, there is a tendency toward sclerosis, especially in young persons

Follis I would say there are exceptions to that These cases of Prof Fanconi's glomerular renal disease, not tubular but primarily glomerular nephritis, pyelonephritis and the late cases of vascular nephritis, they usually start off with osteomalacia which goes on into osteitis fibrosa and may at the end be pure osteitis fibrosa

Rutishauser It is not only in cases of renal diseases but also in cases involving other splanchnic organs particularly in cases of cirrhosis of the liver, that osteotrophic repercussions can be shown at least histologically (splanchnic osteopathies) The histology of these diseases is very complex Forms which lead to macroscopically observable changes are rarely seen

Follis But you do see pure ones?

Rutishauser We examined systematically 265 skeletons which had belonged to persons over thirty years of age We found 20 osteopathies of mixed character, of which 2 were of the fibrous form and 16 were pure osteomalacias Wernly examined 470 skeletons under the same conditions and found 33 osteopathies of the mixed form (renal osteodystrophies) and 24 senile osteomalacias

Follis But where are you on the distribution curve?

Rutishauser It depends on the relative importance of osteoid seams and hyperplasia of the endosteum with remodelling It appears to me to be a matter of definition

Nordin Dr Follis, when you say that the bone was absolutely normal in the 6 cases you studied were you looking for evidence of osteitis fibrosa, as one usually does in cases of hyperparathyroidism? I have the impression that the situation is even more confused, because you can get osteoporosis in these people Could you be sure that in these bits of bone which you got from Dr Howard the trabeculae or the cortex was not a little thin?

Follis I think you would have to make that diagnosis on the roentgenological appearance

Nordin But is it not a fact that in some of these cases where one says there is no bone disease and the alkaline phosphatase is normal the radiologists see a general diffuse osteoporosis? Does histology not confirm that there is an osteoporosis there?

Dent Clinically some of these people have aches and pains in their bones which disappear after the removal of the tumour which suggests that something is going wrong even though you cannot see it radiologically or histologically

Lacroix Among the questions to be dealt with in this general discussion I should like to raise the following Is every part of the

pituitary forms and the primary adrenocortical forms of Cushing's syndrome

Dent In secondary hyperparathyroidism if you then have large parathyroid glands, you always have bone disease There is a very close correlation between the histology and size of the parathyroid and the extent of the osteitis fibrosa In secondary hyperparathyroidism there is this correlation, but in primary there is not The whole thing defeats me

Nassim Are the balances identical?

Dent As far as I know, they are

Nassim May it not be that the target organ is not sensitive to parathyroid hormone and you are just getting an increased vitamin D effect with increased absorption through the intestine?

Dent I like that suggestion very much but I wonder how much truth there is in it In secondary hyperparathyroidism one is up against a different problem If you have large parathyroid glands at autopsy in a patient who died of chronic renal failure, you have osteitis fibrosa generalisata

Nassim That is not comparable in this case where you have got the target organ insensitive

Howard Dr Dent's last sentence is not 100 per cent true either

Dent I am quoting in particular the London Hospital series with 90 cases of renal failure

Armstrong Is it possible that you recognized the cases of hyper parathyroid hyperplasia only in the individuals who had bone disease?

Dent No, because they all come to postmortem

Follis I think it is worth while pointing out that these individuals who do not have any changes grossly or roentgenologically, also do not have them microscopically Through Dr Howard's courtesy we have had a chance to study histologically 6 specimens of the sternum and clavicle and we were giving up the concept that hyperparathyroidism ever caused bone disease, we did not find any changes! I would question the statement that you always see osteitis fibrosa in renal disease

Dent You do if the parathyroid glands are hypertrophic at post mortem

Follis Where are you going to draw the line? We would say that the first change was probably osteomalacia

Dent Yes we would too without enlargement of the parathyroid

Follis In renal disease

Rutishauser Renal osteodystrophies have a very complex histology The majority of serious cases have the appearance of osteo malacia, but there are also many cases which are mixed at the

whole skeleton liable to be absorbed to the same extent in hyperparathyroidism? I suggest that different generations of lamellae might possibly react differently in this disease I think that Prof Engstrom will not agree with me on this point and that he thinks of an absorption cavity as destroying any surrounding bone, irrespective of its age But there are some observations which seem to indicate that if a young osteon is in the way of an extending Haversian space, it is not at once destroyed, it remains unaltered for a while, bulging in the cavity I wonder whether the absorption process in hyperparathyroidism is able to make a similar choice

Nordin It may be relevant to this point of Prof Lacroix that we have seen hyperparathyroidism recently in a boy of 17, who had got bone disease and an alkaline phosphatase of 90, Ca 17, P 2 8, and a palpable tumour Now his iliac crest bone biopsy showed the big osteoid seam of osteomalacia There was osteitis fibrosa as well, but the fact remains that the osteoid seams were very big In the ordinary course of events this would be called osteomalacia He has had his tumour removed and his Ca has come down to 9 There is no doubt about the diagnosis

Follis Could he not be turning on and off?

Nordin He was running at Ca 17 for about 8 months before he came to surgery He must have had 15–20 observations

Dent What were his X rays like?

Nordin He had subperiosteal erosion in the phalanges no actual cysts but what the radiologists regard as osteitis fibrosa in the hands at least certainly thin bone elsewhere

Dent I have seen one case like that but that case had steatorrhoea and was very complicated Dr Nassim has seen a case with slight hyperparathyroidism which was corrected either spontaneously or with vitamin D I am not sure which We think that sometimes in steatorrhoea you get a secondary hyperparathyroidism which gets out of control It presents with a high Ca and a mixed histological picture *We had one case which we think was such a condition*

Fanconi Is it a question of the degree of acidosis if you have osteomalacia or only fibro osteoclasia? I believe that osteomalacia is chiefly due to acidosis whereas fibro osteoclasia is a consequence of hyperparathyroidism

Nordin He had evidence of K deficiency He was not acidotic In fact he was slightly alkalotic He had hypokalaemic alkalosis

Rutishauser Bone biopsies yield excellent information on a well established bone disease In cases of mildly pronounced bone diseases, they can be misleading since the intensity of bone lesions varies strikingly from one place to another

Dent Our pathologists told us it was indistinguishable

Howard Not necessarily I do not think anyone has any proof of this They have made all sorts of pituitary extracts and put them in animals and have found no evidence biochemically or by looking at the parathyroids that anything happens Nicholson wasted three years on that at Johns Hopkins

Nordin In acromegaly you have a high serum phosphate

Dent Not if you have a parathyroid tumour

Rutishauser I examined several cases of multiple endocrine adenomata and particularly pituitary parathyroid syndromes Here it is possible that we are not dealing with endocrine interaction but rather with the expressions of a pleiotrophic gene

Fanconi Has anyone got information about the function of the glomus caroticum? I read somewhere that its function is as the chemoreceptor of Ca metabolism

Rutishauser We had a case of multiple endocrine adenomata in which the first manifestation was a glomus body tumour (44 year old woman) The tumour was incompletely extirpated firstly 6 years and secondly 2 years before her death Following the second operation the patient developed Cushing's syndrome of adrenocortical origin She died suddenly 10 hours after removal of the adrenocortical adenoma Autopsy revealed a typical Cushing's syndrome and no regrowth of the glomus tumour

Bélanger During this symposium several speakers Profs Dallemagne Lacroix and Meyer in particular have mentioned the relationship between organic and inorganic constituents of mineral ized tissues Recently a series of data has been obtained in our laboratory which I believe to be related to that problem With your permission Mr Chairman I should like to present these to this assembly In 1952 autoradiographs were obtained from sections of mineralized bones and teeth soaked for a few hours in a weak solu tion of $H_3{}^{3}PO_4$ (Belanger L F (1953) *J dent Res* 32, 168) Contrary to expectation the pictures attributed to atomic inter exchange have revealed that the areas of most intense radioactivity were the least mineralized This could be explained by greater or lesser ionic transit This simple type of experimentation was repeated with a solution of $^{45}CaCl$ It produced comparable pictures over mineralized bone but this time there appeared to be also a picture of low intensity over the whole cartilaginous epiphyseal plate Sections of demineralized bone tissue were then treated in a similar fashion and they have produced autoradiographs in which the epiphyseal plate stood out sharply as radioactive while the bone tissue was now negative (Fig 1) (Belanger L F (1955) *Proc Soc exp Biol, N Y* 88, 150) At a later stage sections made radioactive by this process were negativated when subsequently incubated in hyaluronidase

have helped me so much that I have thoroughly enjoyed myself and am at least unaware that either of these unfortunate possibilities has occurred

In these few days we have covered a wide field of bone metabolism and growth the structure function and pathology, and we have seen what is going on in different aspects of this field It seems to me that the anatomists and the histologists the people that is who look at bone are making faster progress than the rest of us The biochemists are up against the problem of the sample where to take it from its purity and how to handle it As for the clinicians, they are greatly handicapped by the unfortunate inability to obtain and study pure parathyroid hormone and by their inability to assay it in biological fluids As a result they are making only slow progress, as far as I am aware I do not think we will get very much further until we settle more directly the question of which clinical situations really have got hyperparathyroidism, primary or secondary and which have not

During this week we have had a rare opportunity for meeting many people in closely related fields and of discussing matters as one body In future I am going to feel rather lonely carrying on again by myself and trying to do without some of you However we have a possible solution We can now write to each other more freely when we get into difficulties with some aspects of our work that we do not really under stand I sometimes think that this is the greatest benefit we derive from meetings of this kind

Young human tracheal cartilage became diffusedly radioactive when treated *in vitro* with ^{45}Ca On the other hand, the trachea of an old woman (Fig 2) produced autoradiographs of considerably less ^{45}Ca intake localized in the periphery, and also present over the cells this time

It appears from these findings that the ^{45}Ca uptake of trachea is related to its content of chondroitin sulphate and also that this substance in old age, is secreted less abundantly and accumulates in the cells This is the time when the trachea becomes mineralized

Finally, we have had the opportunity recently to observe ^{45}Ca *in vitro* uptake in bones and teeth of pigs which had been fed a large dose of NaF for several weeks (Bélanger L F, Lotz W E, Visek W J, and Comar C L (1956) *Amer J Anat*, in press) In these, a wide band of osteoid at the border of newly formed trabeculae and also wide irregular predentine of recent growth produced a record of ^{45}Ca intake On the other hand, micro incinerated sections of demineralized bones and teeth from these fluorinated pigs revealed a progressive accumulation of ash of which only a portion had the solubility of CaF_2 What about the rest of it? Could it represent some organic form of Ca combination which under normal conditions would have a quick turnover a rapid intermediate step between ionized Ca and mineral Ca? Could the poor mineralization of bone and dentine in fluorine intoxication be partly due to a vital interference with this mechanism resulting in the accumulation of the intermediate organic product? At any rate I believe that, *in vitro* ^{45}Ca has revealed a definite tissue affinity related to chondroitin sulphate which may well be of physiological significance

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Dent In these closing remarks I should like to take the opportunity to thank again the Ciba Foundation and Dr Wolstenholme with his charming and efficient staff for this most successful meeting I should like to thank also the members of the conference themselves When I took on this job of chairman I thought it would inevitably make me lose a few friends and perhaps make one or two new enemies but in fact you

PLATE I

FIG 1 (Bélanger) Lower extremity of femur and upper extremities of tibia and fibula of adult female rat ^{45}Ca is present almost exclusively in epiphyseal plate and cartilaginous core of bone spicules Inverted and stained autoradiograph × 15 (Reproduced by permission of *Proc Soc exp Biol N Y*)

FIG 2 (Bélanger) Portion of tracheal cartilage from a woman aged 68 years The ^{45}Ca intake is visible only over the large lacunae and immediate vicinity of those close to surface Unstained integrated autoradiograph × 124 (Reproduced by permission of *Proc Soc exp Biol N Y*)

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SUBJECT INDEX

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